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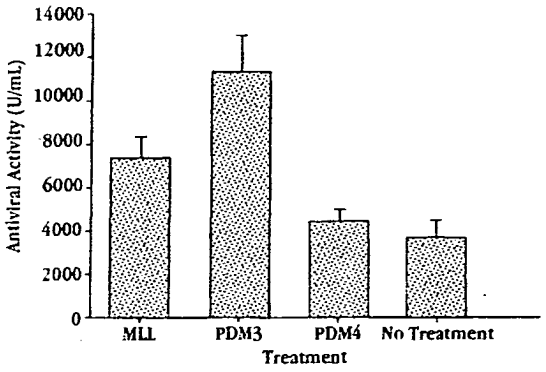
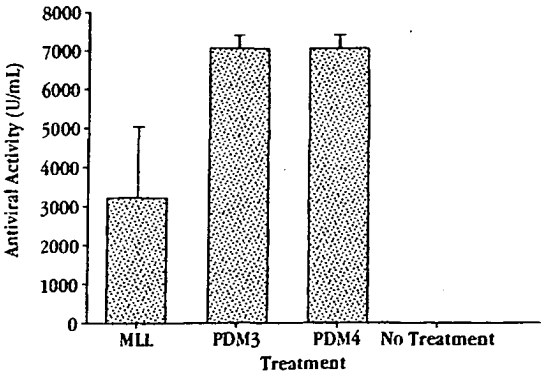
(54) Title: COMPOSITIONS FOR TRANSDERMAL AND TRANSMUCOSAL ADMINISTRATION OF THERAPEUTIC AGENTS





(57) Abstract: A composition for transdermal or transmucosal administration of a therapeutic agent is described. Penetration of the agent across the skin or mucosa is achieved in the presence of an acylated amino acid selected to enhance the agent to be administered. In some embodiments, a liposomal carrier vehicle is included in the composition. Also disclosed are methods for administration and for selection of an acylated amino acid to optimize transdermal or transmucosal administration of a selected agent.

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COMPOSITIONS FOR TRANSDERMAL AND TRANSMUCOSAL ADMINISTRATION OF THERAPEUTIC AGENTS

Field of the Invention

The present invention relates to compositions and methods for administration of therapeutic agents across the skin or mucosa. In particular, the invention relates to acylated amino acid compounds for transdermal and transmucosal administration of macromolecules, such as polypeptides and proteins.

Background of the Invention

The skin provides an appealing site for the noninvasive entry of drugs into the body. Delivery of drugs through the skin for a systemic effect, or transdermal delivery, has become a conventional and recognized approach for small, lipophilic molecules, such as nitroglycerin and nicotine. However, for hydrophilic and macromolecular drugs, such as peptides and proteins, transdermal and transmucosal administration has been less successful.

One approach for transdermal administration of proteins and other macromolecues is iontophoretic delivery. Electroporation has also been described for use in achieving transdermal delivery of compounds. While these approaches have met with some success, a passive method capable of delivering sufficient amounts of peptides, proteins, or polynucleotides into or through the skin is still desired.

The transdermal and transmucosal route of administration of proteins offers several advantages. Since these compounds have short half-lives, the continuous mode of transdermal administration derives maximum therapeutic benefits. The skin is also very low in proteolytic activity, as compared to, for example, the oral route of administration, so that degradation at the administration site is reduced. Additionally, a drug absorbed transdermally bypasses the hepatic circulation, avoiding another major site of potential degradation.

However appealing the transdermal route of administration may be, attempts at administering the compounds traditionally considered difficult or impossible to deliver transdermally have not been successful, as the skin presents a formidable barrier to penetration. The uppermost layer of the skin, the stratum corneum, serves as the primary barrier to absorption of substances coming into contact with the skin. The stratum corneum consists of dead, flattened keratin-filled cells, corneocytes, in a lipid matrix. Each

corneocyte is bounded by a thick, proteinaceous envelope with rough fibrous protein (keratin) as the main component. The intercellular spaces are filled with broad multiple lamellae of lipids, primarily ceramides, cholesterol, fatty acids, and cholesteryl esters. Human skin is 2-3 mm thick, and the stratum corneum over most of the body is 15 μm thick.

Summary of the Invention

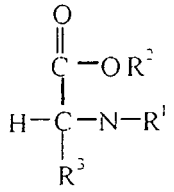
Accordingly, it is an object of the invention to provide a composition for transdermal and/or transmucosal administration of a compound.

It is another object of the invention to provide a composition for transdermal administration of a macromolecule, such as a polypeptide or a protein.

In one aspect, the invention includes a composition for transdermal or transmucosal administration composed of a therapeutic agent and an acylated amino acid, wherein application of the composition to the skin is effective to achieve enhanced uptake of the therapeutic agent when compared to transdermal uptake of the therapeutic agent alone, that is, in the absence of the acylated amino acid.

In one embodiment, the composition further comprises a suspension of liposomes. The therapeutic agent and/or the acylated amino acid, in other embodiments, is entrapped in the liposomes. The liposomes can have an aqueous inner core or an oil-in-water emulsion in liposomes' central compartment.

The acylated amino acid, in a preferred embodiment, is an acylated amino acid represented by the formula:



wherein R^1 is an acyl group having from 1-20 carbons, R^2 is hydrogen or an alkyl group, and R^3 corresponds to a modified or unmodified R group of a selected amino acid.

The therapeutic agent can be virtually any compound, and in preferred embodiments in a macromolecule such as a peptide, protein, or nucleic acid. Interferons, interleukins, and insulin are exemplary agents.

In another aspect, the invention includes a method for administering a therapeutic agent to a subject. The method includes preparing a composition comprised of a

therapeutic agent and an acylated amino acid, as describe above, and administering the composition transdermally to the subject. Administration of the composition is effective to achieve a concentration of the agent in the blood sufficient for therapy.

In yet another aspect, the invention includes a method for selecting a composition for therapeutic transdermal administration of a macromolecule.

In still another aspect, the invention includes a transdermal delivery device, comprising; (i) a suspension comprised of liposomes, an acylated amino acid, and a macromolecular therapeutic agent; (ii) a reservoir adapted to retain the suspension and adapted for release of lipid vesicles therefrom; and (iii) means for affixing the device to a subject for transdermal administration of said agent.

In yet another aspect, the invention includes an interferon-α composition comprising biphasic lipid vesicles comprised of (i) a lipid bilayer comprising a phospholipid and a fatty acylated amino acid; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, where the oil-in-water emulsion is stabilized by a surfactant; and (iii) interferon-α entrapped in the vesicles. The composition when applied to the skin of a subject is effective to administer a therapeutically effective amount of interferon-α.

The acylated amino acid, in one embodiment, is selected to achieve dermal administration of interferon-α for treatment of a local, topical condition.

In another embodiment, the acylated amino acid is effective to achieve transdermal administration of interferon-α.

In a preferred embodiment, R^2 in the acylated amino acid is $((CO)C_{19}H_{39})$. In particular, when R^2 is $((CO)C_{19}H_{39})$ the amino acid is serine or threonine. Another preferred acylated amino acid is monolauroyl lysine.

In still another embodiment, the oil-in-water emulsion in the biphasic lipid vesicles further comprises a fatty alcohol. For example, the fatty alcohol can have between about 8-24 carbon atoms. In another embodiment, the oil-in-water emulsion further comprises a triglyceride, such as pharmaceutically-acceptable oil, such as canola oil and olive oil.

In yet another embodiment, the oil-in-water emulsion is further comprised of a fatty glyceride dispersed in the water phase and stabilized by the surfactant. Such a fatty glyceride can be, for example, glycerol monostearate.

The lipid bilayer of the vesicles can further comprise of a sterol.

In another aspect, the invention includes a composition for administration of interferon-α, comprising biphasic lipid vesicles comprised of (i) a lipid bilayer comprised of a phospholipid and an amino acid acylated; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, where the oil-in-water emulsion is comprised of a triglyceride that is dispersed in a water phase containing a fatty alcohol and that is stabilized by a surfactant; and (iii) interferon-α entrapped in the vesicles. The composition when applied to the skin of a subject is effective to administer a therapeutically effective amount of interferon-α.

In still another aspect, the invention includes a method of administering a therapeutically effective amount of interferon-α to a subject, comprising preparing biphasic lipid vesicles as described above. The biphasic lipid vesicles are then contacted with the skin of a subject for transdermal or dermal delivery, depending on the selected acylated amino acid.

In a further aspect, the invention includes a method of treating human papilloma virus in a subject. The method includes preparing biphasic lipid vesicles as described above and contacting the biphasic lipid vesicles with the skin of a subject, or more preferably, applying the lipid vesicles to the site of the infection.

In one aspect, the invention includes a composition for transdermal administration of insulin. The composition includes biphasic lipid vesicles comprising (i) a lipid bilayer comprised of a phospholipid and a fatty acylated amino acid; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, where the oil-in-water emulsion is comprised of a triglyceride dispersed in a water phase and stabilized by a surfactant; and (iii) insulin entrapped in said vesicles. The composition when applied to the skin of a subject is effective to administer a therapeutically effective amount of insulin.

In one embodiment, the lipid bilayer of the biphasic lipid vesicles is further comprised of a sterol.

In another embodiment, the acylated amino acid is an acylated lysine. One preferred acylated lysine is Nα-capryloyl-Nε lauroyl L-lysine ethyl ester (PDM27).

In another embodiment, the triglyceride in the lipid vesicles is a natural oil, such as canola or olive oil.

In another aspect, the invention includes a composition for transdermal administration of insulin, comprising biphasic lipid vesicles comprised of (i) a lipid

bilayer comprised of a phospholipid and a fatty acylated lysine compound; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, where the oil-in-water emulsion is comprised of a triglyceride dispersed in a water phase and stabilized by a surfactant; and (iii) insulin entrapped in said vesicles. The composition when applied to the skin of a subject is effective to administer a therapeutically effective amount of insulin.

In one embodiment of this aspect, the surfactant is a cationic phospholipid.

In yet another aspect, the invention includes a composition for transdermal administration of insulin, comprising biphasic lipid vesicles comprised of (i) a lipid bilayer comprised of a phospholipid and Nα-capryloyl-Nε-lauroyl L-lysine ethyl ester; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, where the oil-in-water emulsion is comprised of a triglyceride dispersed in a water phase and stabilized by a surfactant; and (iii) insulin entrapped in said vesicles. The composition when applied to the skin of a subject is effective to administer a therapeutically effective amount of insulin.

In still another aspect, the invention includes a method of administering a therapeutically effective amount of insulin to a subject, comprising preparing biphasic lipid vesicles as described above and contacting the biphasic lipid vesicles with the skin of a subject.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1A is a bar graph showing the antiviral activity in serum taken from guinea pigs after transdermal administration of IFN-α from biphasic lipid vesicles containing IFN-α and monolauroyl lysine (MLL), N-eicosanoyl-L-serine (PDM3) or N-eicosanoyl threonine (PDM4) entrapped in the lipid vesicles.

Fig. 1B is a bar graph showing the antiviral activity in skin homogenates prepared from the skin of guinea pigs after transdermal administration of IFN-α from biphasic lipid vesicles containing IFN-α and monolauroyl lysine (MLL), N-eicosanoyl-L-serine (PDM3) or N-eicosanoyl threonine (PDM4) entrapped in the lipid vesicles.

Figs. 2A-2C are graphs showing the antiviral activity in skin biopsy samples taken

from human subjects after transdermal administration of IFN-α from biphasic lipid vesicles containing monolauroyl lysine and IFN-α at dosage levels of 5 MU, 15 MU, and 40 MU entrapped in the lipid vesicles. The Y-axis shows the individual's initials and the numbers above each bar are the fold increase over each individual's respective untreated skin sample.

Figs. 3A-3C are graphs showing IFN-α concentration as pg/mg protein in skin homogenate prepared from samples taken from human subjects after transdermal administration of IFN-α from biphasic lipid vesicles containing monolauroyl lysine and IFN-α at dosage levels of 5 MU, 15 MU, and 40 MU entrapped in the lipid vesicles. The Y-axis shows the individual's initials and the numbers above each bar are the fold increase over each individual's respective untreated skin sample.

Figs. 4A-4C are graphs showing the antiviral activity in serum samples taken from human subjects after transdermal administration of IFN-α from biphasic lipid vesicles containing monolauroyl lysine and IFN-α at dosage levels of 5 MU, 15 MU, and 40 MU entrapped in the lipid vesicles. The Y-axis shows the individual's initials and the numbers above each bar are the fold increase over each individual's respective untreated skin sample.

Figs. 5A-5C are graphs showing the 2-5A synthetase enzyme activity in serum samples taken from human subjects after transdermal administration of IFN-α from biphasic lipid vesicles containing monolauroyl lysine and IFN-α at dosage levels of 5 MU, 15 MU, and 40 MU entrapped in the lipid vesicles. The Y-axis shows the individual's initials and the numbers above each bar are the fold increase over each individual's respective untreated skin sample.

Fig. 6 shows the serum insulin concentration in a diabetic rat following subcutaneous injection of insulin (1 mg).

Fig. 7 shows blood glucose levels, in mmol/L, as a function of time, in hours, in rats treated with transdermally administered insulin from biphasic lipid vesicles containing insulin and the acylated amino acid PDM27 (open triangles). The dosage applied was 200 mg of the biphasic lipid vesicle formulation having 50 mg insulin/mg formulation. The blood glucose levels of non-diabetic, healthy, untreated rats (solid triangles) and of diabetic, untreated rats (open circles) are also shown.

Fig. 8 shows the change in blood glucose levels, in mmol/L, as a function of time, in hours, in rats treated with transdermally administered insulin from biphasic lipid vesicles containing insulin and the acylated amino acid PDM_{27} (open triangles). The dosage applied

was 1000 mg of the biphasic lipid vesicle formulation having 10 mg insulin/mg formulation. The blood glucose levels of non-diabetic, healthy, untreated rats (solid triangles) and of diabetic rats treated with 1 mg insulin administered subcutaneously (open circles) are also shown.

Fig. 9 is a bar graph showing the duration of response to transdermally (dotted bars) and subcutaneously (lined bars) administered insulin. The insulin administered subcutaneously at a dose of 28 U was in the form of a saline solution. The insulin administered at a dose of 140 U and 280 U, both transdermally and subcutaneously, was in the form of biphasic lipid vesicles containing insulin.

Detailed Description of the Invention

I. Definitions

"Acylated amino acid" refers to an amino acid modified at one or more amine groups with an acylating agent that reacts with an amine group.

"Amino acid" refers to any carboxylic acid having at least one free amine group, including naturally-occurring and synthetic amino acids.

"Dermal" as used herein intends transport of an agent across the stratum corneum and into the viable epidermis for treatment of a topical skin disorder that responds to local, non-systemic administration of an agent. It will be appreciated that some of the agent intended for dermal therapy may be transdermally administered, however typically not in an amount sufficient for therapy.

"Fatty-acylated amino acid" refers to an acylated amino acid where the acyl group has more than about 8 carbon atoms.

"Interferon-alpha" is abbreviated herein as "IFN-α" and intends all of the known and unknown subtypes including but not limited to IFN-α_1, IFN-α_2, IFN-α_4, IFN-α_5, IFN-α_8, IFN-α_7, IFN-α_{10}, IFN-α_{14}, IFN-α_{17}, IFN-α_{21}, and their variants including but not limited to IFN-α_{1a}, IFN-α_{2a}, IFN-α_{2b}, IFN-α_{2c}, IFN-α_{4a}, IFN-α_{4b}, IFN-α_{7a}, IFN-α_{7b}, IFN-α_{7c}, IFN-α_{8b}, IFN-α_{10a}, IFN-α_{14a}, IFN-α_{14b}, IFN-α_{14c}, IFN-α_{17b}, IFN-α_{21a}, IFN-α_{21b}.

An "R group of an amino acid" as used herein refers to the R group in the general structure [(COOH)CHNH$_2$(R)], where R represents the moiety that individualizes each amino acid. R is selected from H (glycine), CH_3 (alanine), CH_2OH (serine) CH_2COOH (aspartic acid), $(CH_2)_3NHC(NH)NH_2$ (arginine), $CH(CH_3)_2$ (valine), $CHOH(CH_3)$

(threonine), CH_2CH_2COOH (glutamic acid), $CH_2CH(CH_3)_2$ (leucine), CH_2SH (cysteine), $CH_2(C(NH)(CH)(N)(CH))$ (histidine), $(CH_2)_4NH_2$ (lysine), $CH(CH_3)CH_2CH_3$ (isoleucine), $CH_2(C_6H_4)OH$ (tyrosine), $CH_2(CONH_2)$ (asparagine), $(CH_2)_2SCH_3$ (methionine), $(CH_2)_3$ (proline), $CH_2C_8NH_6$ (tryptophan), $CH_2(C_6H_5)$ (phenylalanine) and $(CH_2)_2(C)(O)(NH_2)$ (glutamine).

"Therapeutically effective amount" intends an amount of a therapeutic agent sufficient to reduce the symptoms associated with a disease or condition and/or lessen the severity of the disease or condition. The condition can be a topical, local condition, such as genital warts, or a more pervasive condition, such as a viral infection or diabetes. A therapeutically effective amount is understood to be in context to the condition being treated, where the actual effective amount is readily discerned by those of skill in the art.

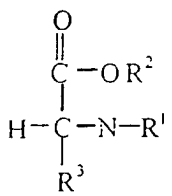
"Transdermal" as used herein intends transport of an agent across the stratum corneum for introduction into systemic circulation.

II. Composition Components

As discussed above, the compositions of the invention are comprised of a therapeutic agent and an acylated amino acid. The two components are placed in a selected carrier vehicle for administration transdermally or transmucosally. The acylated amino acid, the therapeutic agent and the carrier vehicles will now be described.

1. Acylated Amino Acids

Tables 1A-1F show exemplary acylated amino acids for use as absorption promoters in the compositions of the present invention. Generally, the acylated amino acid compounds are represented by the structure X-CO-A, where X is an aliphatic hydrocarbon group, an aryl-substituted lower hydrocarbon or an aromatic hydrocarbon group, each of which may optionally be substituted, and A is an amino acid residue which may optionally be substituted. Alternatively, the acylated amino acids are represented by the general formula:



wherein R^1 is typically an acyl group having from 1-20 carbons, R^2 is hydrogen or a lower

alkyl. and R^3 corresponds to the R group of the selected amino acid. In some cases R^3 includes an amino group which can be acylated. such as when R^3 is lysine. arginine. or glutamine. and compounds PDM5 and PDM17 in Table 1A are exemplary.

Table 1A

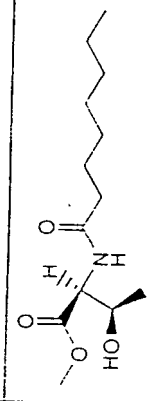
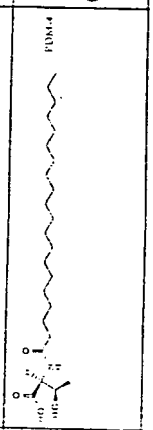
Chemical Name	Code	Chemical Structure	Properties
N-capryloyl-L- threonine methyl ester	PDM1		Mwt: 259.34 Molecular Formula: $C_{13}H_{25}NO_4$
N-eicosanoyl-L-serine	PDM3		Mwt: 399.61 Molecular Formula $C_{23}H_{45}NO_4$
N-eicosanoyl threonine	PDM4		Mwt: 413.62 Molecular Formula: $C_{24}H_{47}NO_4$

Table 1A (continued)

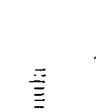
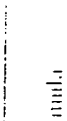
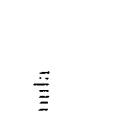
Chemical Name	Code	Chemical Structure	Properties
Nα- capryloyl-Nε-lauroyl- L-lysine methyl ester	PDM15		Mwt 468.71 Molecular Formula $C_{25}H_{48}N_2O_4$
Nα-palmitoyl-N ε Lauroyl-L-lysine methyl ester	PDM17		Mwt 580.93 Molecular Formula $C_{35}H_{68}N_2O_4$
Nα lauroyl- Nε Lauroyl- L-lysine methyl ester	PDM18		Mwt 524.82 Molecular Formula $C_{31}H_{60}N_2O_4$
Nε - lauroyl-L lysine ethyl ester	PDM19		Mwt 356.54 Molecular Formula $C_{20}H_{40}N_2O_4$

Table 1B

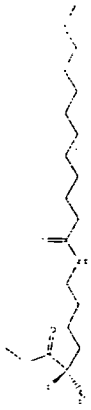
Chemical Name	Code	Chemical Structure	Properties
Nε - lauroyl-L lysine methyl ester	PDM20		Mwt: 342.52 Molecular Formula $C_{19}H_{38}O_3$
Nα-capryloyl- Nε lauroyl L-lysine ethyl ester	PDM27		Mwt: 482.74 Molecular Formula: $C_{28}H_{54}N_2O_4$
Nα Maleoyl-Nε lauroyl-L-lysine ethyl ester	PDM29		Mwt: 454.60 Molecular Formula: $C_{24}H_{42}N_2O_6$

Table 1B (continued)

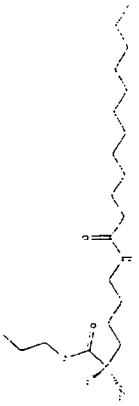
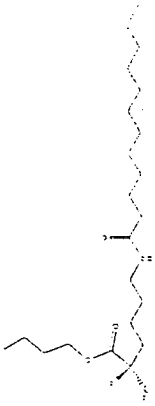
Chemical Name	Code	Chemical Structure	Properties
Nε Lauroyl L-lysine-n-propyl ester	PDM-41		Mwt 370.57 Molecular Formula $C_{21}H_{42}N_2O_3$
Nε- Lauroyl-L-Lysine-n-butyl ester	PDM-42		Mwt 384.60 Molecular Formula $C_{22}H_{44}N_2O_3$
Nε- Lauroyl-L-lysine-iso-amyl ester L-isomer	PDM-43		Mwt 398.62 Molecular Formula $C_{23}H_{46}N_2O_3$

Table 1C

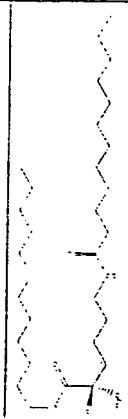
Chemical Name	Code	Chemical Structure	Properties
Nε - Lauroyl - L-lysine-dodecyl ester	PDM45		Mwt: 496.81 Molecular Formula: $C_{30}H_{60}N_2O_3$
Nα - capryloyl-N-ε lauroyl-L-lysine-n-propyl ester	PDM46		Mwt: 496.77 Molecular Formula: $C_{29}H_{56}N_2O_4$
Nα - capryloyl-N-ε lauroyl-L-lysine-n-butyl ester	PDM47		Mwt: 510.79 Molecular Formula: $C_{30}H_{58}N_2O_4$

Table 1C (continued)

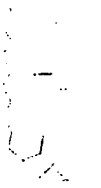
Chemical Name	Code	Chemical Structure	Properties
Nα - capryloyl-Nε-Lauroyl -L-lysine- iso-amyl ester + isomer	PDM49		Mwt 524.82 Molecular Formula $C_{30}H_{60}N_2O_4$
Nα - capryloyl-N-ε lauroyl-L-lysine-n dodecyl ester	PDM50		Mwt 623.1 Molecular Formula $C_{38}H_{72}N_2O_4$
Nα-maleoyl-Nε- lauroyl-L-lysine- dodecyl ester	PDM51		Mwt 594.87 Molecular Formula $C_{34}H_{62}N_2O_6$

Table 1D

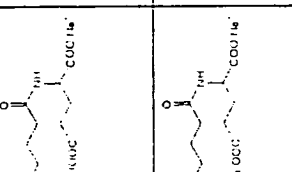
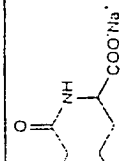
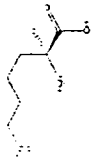
Chemical Name	Brand Name	Code	Chemical Structure	Properties
Sodium N-Stearoyl-L-Glutamate	Amisoft HS-11 (Ajinomoto USA Inc., Teaneck, NJ)	AG5	 R = stearoyl	Mwt: 435.57 Molecular Formula: $C_{23}H_{42}NNaO_5$
Disodium Stearoyl-L-Glutamate	Amisoft HS-21 (Ajinomoto USA Inc., Teaneck, NJ)	AG6	R - stearoyl	Mwt: 457.55 Molecular Formula: $C_{23}H_{41}NNa_2O_5$
Sodium Lauroyl-L-Glutamate	Amisoft LS-11 (Ajinomoto USA Inc., Teaneck, NJ)	AG7	 R - lauroyl	Mwt: 351.41 Molecular Formula: $C_{17}H_{30}NNaO_5$
Lauroyl lysine	Amihope LL (Ajinomoto USA Inc., Teaneck, NJ)	MLL		Mwt: 328.49 Molecular Formula: $C_{18}H_{36}N_2O_3$
Tea - Lauroyl Animal Collagen Amino Acids	Aminofoam C (Croda Canada, Toronto, ON)	AFC	--	Mwt: 550
Tea - Lauroyl Keratin Amino Acids	Aminofoam K (Croda Canada, Toronto, ON)	AFK	--	Mwt: 550

Table 1E

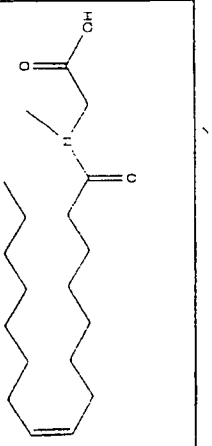
Chemical Name	Brand Name	Code	Chemical Structure	Properties
Cocyl Sarcosine	Hamposyl C (Hampshire Chemical Corp., Lexington, MA)	HC	R - CO - N - CH_2 - COOH CH_3 R-cocoyl	Mwt: 280
Lauroyl Sarcosine	Hamposyl L (Hampshire Chemical Corp., Lexington, MA)	HL		Mwt: 271.40 Molecular Formula: $C_{15}H_{29}NO_3$
Oleyl Sarcosine	Hamposyl O (Hampshire Chemical Corp., Lexington, MA)	HO		Mwt: 325.49 Molecular Formula: $C_{19}H_{35}NO_3$

Table 1E (continued)

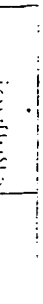
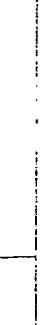
Chemical Name	Brand Name	Code	Chemical Structure	Properties
Myristoyl Sarcosine	Hamposyl M (Hampshire Chemical Corp., Lexington, MA)	IIM		Mwt: 299.45 Molecular Formula $C_{17}H_{33}NO_3$
N,N Dipalmitoyl Lysine	-- (Cananmino Inc Ottawa, ON)	DPL		Mwt: 623.01 Molecular Formula $C_{38}H_{74}N_2O_5$
N Cinnamoyl Phenyl alanine	-- (Cananmino Inc Ottawa, ON)	CPh		Mwt: 295.37 Molecular Formula $C_{18}H_{17}NO_3$

Table 1F

Chemical Name	Brand Name	Code	Chemical Structure	Properties
N Myristoyl Glycine	– (Canamino Inc. Ottawa, ON)	MG		Mwt: 285.43 Molecular Formula: $C_{16}H_{31}NO_3$
N-Acetyl-L-Cysteine	– (Canamino Inc. Ottawa, ON)	AC		Mwt: 239.27 Molecular Formula: $C_5H_9NO_2$
Cocoyl Glutamate	Amisoft CA (Ajinomoto USA Inc., Teaneck, NJ)	AG1		Mwt: ~400
Potassium Cocoyl Glutamate	Amisoft CK11 (Ajinomoto USA Inc., Teaneck, NJ)	AG2		Mwt: ~400
Tea Cocoyl Glutamate	Amisoft CT-12 30% aqueous solution (Ajinomoto USA Inc., Teaneck, NJ)	AG3		Mwt: ~400
Sodium Cocoyl Glutamate & Sodium Hydrogenated Tallow-Glutamate	Amisoft GS-11 (Ajinomoto USA Inc., Teaneck, NJ)	AG4		Mwt: 420

The preferred naturally occurring α-amino acids for use in the invention are alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. More preferred amino acids are lysine, threonine, serine, glycine, cysteine and glutamine.

Modification of α-amino acids by acylating at least one free amino group is readily performed using an acylating agent that reacts with a free amino group. The compounds

listed in Tables 1A-1C were synthesized for studies conducted in support of the present invention and synthetic reaction schemes for the compounds are described in Examples 1-19. The compounds listed in Tables 1D-1F are commercially available from the vendors indicated in the second column of the tables.

2. Therapeutic Agent

The therapeutic agent to be used in the present invention is not limited, and includes ordinary pharmaceuticals, water-soluble and non-water-soluble compounds, low molecular weight compounds, high molecular weight compounds and macromolecules, such as peptides, proteins and protein hormones.

In a preferred embodiment, the therapeutic agent is a macromolecule, by which is meant a therapeutic agent having a molecular weight above about 500 Daltons, more preferably above 1000 Daltons, still more preferably above 2000 Daltons. Exemplary macromolecules include proteins, including but not limited to insulin, somatostatin, calcitonin, angiotensin, secretin, parathhryoid hormone, granulocyte colony-stimulation factor, glucocerebrosidase, rhDNase, somatotropin, interferons, rFactor VIII, interleukins, erythropoietin, growth factors, growth hormones, LHRH analogues, and the like.

3. Carrier Vehicle

The composition of the invention includes a carrier vehicle for application of the acylated amino acid and therapeutic agent to the skin or mucosa. The carrier vehicle can take the form of a simple aqueous-based solution, a water-in-oil emulsion, an oil-in-water emulsion or a pharmaceutically acceptable solvent, such as polyethylene glycol or polypropylene glycol.

It will be appreciated that the pH of the carrier vehicle can be adjusted to alter the ionization state of the therapeutic agent and/or the acylated amino acid. The ionization can have an effect on the skin penetration rate, thus, can be optimized for maximum penetration.

In one embodiment, the carrier vehicle takes the form of a suspension of liposomes. The liposomes can be small unilamellar vesicles or multilamellar vesicles, and a variety of techniques, such as those detailed by Szoka, (*Ann. Rev. Biophys. Bioeng.* 9:467 (1980)). Multilamellar vesicles (MLVs) can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of liposome-forming lipids of the type described below are dissolved in a suitable organic solvent which is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs.

typically with sizes between about 0.1 to 10 microns.

The liposome-forming lipid components necessarily include a vesicle-forming lipid, by which is meant an amphipathic lipid having a hydrophobic tail and a head group which can form spontaneously into bilayer vesicles in water. The vesicle-forming lipids are preferably ones having two hydrocarbon chains, typically acyl chains, and where the head group is either polar or nonpolar. There are a variety of synthetic vesicle-forming lipids and naturally-occurring vesicle-forming lipids suitable for use, such as phospholipids, which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, and sphingomyelin, where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. These lipids can be obtained commercially or prepared according to published methods.

In addition to the vesicle-forming lipid component, the lipid vesicles of the present invention can include other lipid components capable of being stably incorporated into lipid bilayers, with their hydrophobic moieties in contact with the interior, hydrophobic region of the bilayer membrane, and their polar head groups oriented toward the exterior, polar surface of the membrane. For example, glycolipids, ceramides and sterols, such as cholesterol, coprostanol, cholestanol and cholestane, long chain fatty acids (C_{16} to C_{22}), such as stearic acid, can be incorporated into the lipid bilayer. Other lipid components that may be used include fatty amines, fatty acylated proteins, fatty acylated peptides, oils, fatty alcohols, glyceride esters, petrolatum and waxes. Specific examples of preferred lipids include beeswax, glyceryl stearate, cetyl alcohol, myristyl myristate, and cetyl palmitate.

A cationic lipid can also be used in the lipid components of the liposomes. Cationic lipids also have a lipophilic moiety, such as a sterol, an acyl or diacyl chain, yet the lipid has an overall net positive charge. Preferably, the head group of the lipid carries the positive charge. Exemplary cationic lipids include 1,2-dioleyloxy-3-(trimethylamino) propane (DOTAP); N-[1-(2,3,-ditetradecyloxy)propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide (DMRIE); N-[1-(2,3,-dioleyloxy)propyl]-N,N-dimethyl-N-hydroxy ethylammonium bromide (DORIE); N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA); 3β[N-(N',N'-dimethylaminoethane) carbamoly] cholesterol (DC-Chol); and dimethyldioctadecylammonium bromide (DDAB). The cationic vesicle-forming lipid may also be a neutral lipid, such as

dioleoylphosphatidyl ethanolamine (DOPE) or an amphipathic lipid, such as a phospholipid, derivatized with a cationic lipid, such as polylysine or other polyamine lipids. For example, the neutral lipid (DOPE) can be derivatized with polylysine to form a cationic lipid.

4. Biphasic Lipid Vesicles

In another embodiment, the carrier vehicle takes the form of lipid vesicles having an oil-in-water emulsion incorporated into the central core compartment of the vesicles and between the lipid bilayers. These vesicles are referred to herein as "biphasic lipid vesicles". Such biphasic lipid vesicles have been described, for example, in PCT Publication Nos. WO 95/03787, WO 99/11247 and in U.S. Patent No. 5,853,755.

A. Lipid Component

Biphasic lipid vesicles are prepared from a selected lipid composition comprised of one or more lipids. The composition will include at least one vesicle-forming lipid, described above. In addition to the vesicle-forming lipid component, the biphasic lipid vesicles can further include other lipid components capable of being stably incorporated into lipid bilayers. For example, glycolipids, ceramides and sterols, such as cholesterol, coprostanol, cholestanol and cholestane, long chain fatty acids (C_{16} to C_{22}), such as stearic acid, can be incorporated into the lipid bilayer. Other lipid components that may be used include fatty amines, fatty acylated proteins, fatty acylated peptides, oils, fatty alcohols, glyceride esters, petrolatum and waxes. A skin permeation enhancer, such as an acylated amino acid, can be included as part of the lipid components of the lipid vesicle.

Typically, the lipid vesicles include between about 1-40% of vesicle-forming lipid, more preferably from about 5-25% (percentages are weight percentages based on the total lipid vesicle composition, including the oil-in-water emulsion phase described below). The hydrophilic solvent other than water typically constitutes between 1-15% of the liposome, and the acylated amino acid constitutes between 0.1-5%. Cholesterol, or other sterol, when added, is typically in the 1-10% range.

B. Oil-In-Water Emulsion

As noted above, the biphasic lipid vesicles include an oil-in-water emulsion entrapped in the vesicles' aqueous spaces. The oil-in-water emulsion is comprised of

water, a selected lipophilic, hydrophobic component, and a surfactant. The oil-in-water emulsion is one having water as the continuous phase and the lipophilic component as the dispersed phase. The surfactant serves to stabilize the emulsion, and, during formation of the emulsion, it is added to either the water phase or the lipophilic, oil phase, depending on the hydrophilic-lipophilic balance (HLB) of the surfactant. Typically, the surfactant is mixed with the water and this mixture is added to the oily lipohpilic phase for homogenization and formation of the emulsion.

In a preferred embodiment, the stabilizing surfactant is other than a vesicle-forming lipid, *e.g.*, the surfactant is one which does not spontaneously form lipid bilayers. The oil-in-water emulsion is stable by virtue of the oil droplets in the dispersed phase being surrounded by the surfactant. That is, the hydrophilic portion of each surfactant molecule extends into the aqueous phase of the emulsion and the hydrophobic portion is in contact with the lipophilic droplet. If the emulsion is not surfactant-stabilized prior to contact with the vesicle-forming lipids, the vesicle-forming lipids may act to first stabilize the emulsion rather than form lipid bilayers around the oil-in-water emulsion.

Surfactants suitable for formation of the oil-in-water emulsion are numerous, including both cationic, anionic and nonionic or amphoteric surfactants. In one embodiment, the preferred surfactant is a cationic surfactant, such as linoleamidopropyl propylene glycol-dimonium chloride phosphate, cocamidopropyl propylene glycol-dimonium chloride phosphate and stearamido propylene glycol-dimonium chloride phosphate. These are synthetic phospholipid complexes commercially available from DEBRO (Mississauga; Ontario, Canada) sold under the tradenames Phospholipid EFA™, Phospholipid SV™ and Phospholipid SVC™, respectively.

Exemplary anionic surfactants include acylglutamates, such as triethanolamine-cocoyl glutamate, sodium lauroyl glutamate, sodium hydrogenated tallow glutamate and sodium cocoyl glutamate.

Exemplary nonionic surfactants include naturally derived emulsifiers, such as polyethyleneglycol-60 almond glycerides, avocado oil diethanolamine, ethoxylated jojoba oil (polyethyleneglycol-40 jojoba acid and polyethyleneglycol-40 jojoba alcohol); polyoxyethylene derivatives, such as polyoxyethylene-20 sorbitan monooleate and polyoxythethylene-20 sorbitan monostearate; lanolin derivatives, such as polychol 20 (LANETH 20) and polychol 40 (LANETH 40); and neutral phosphate esters, such as

polypropyleneglycol-cetyl ether phosphate and diethanolamine oleth-3 phosphate.

The oil component of the emulsion can be selected from a variety of lipophilic compounds, including natural and synthetic triglycerides, fatty glycerides, solid and semi-solid waxes and mixtures thereof. In a preferred embodiment, the primary oil component is a triglyceride selected from synthetic and natural oils, such as olive oil, canola oil, sunflower oil and other oils recited in Col. 12, lines 20-42 of U.S. Patent No. 5,854,755, which is herein incorporated by reference. A preferred fatty glyceride is glycerol monostearate and a preferred wax is beeswax. As will be described below, biphasic lipid vesicles prepared in support of the invention included as the primary oil component a triglyceride, with a fatty glyceride and a wax added as minor components.

In one preferred embodiment, the oil-in-water emulsion further includes a fatty alcohol, $C_nH_{n+2}O$, where n is from 2-24, more preferably from 8-24. In a preferred embodiment, the fatty alcohol is cetyl alcohol ($C_{16}H_{34}O$) or stearyl alcohol ($C_{18}H_{38}O$). The fatty alcohol is typically added to the oil phase prior to homogenization.

The oil-in-water emulsion can also include other components, such as antimicrobial agents or preservatives. Typically, these agents are admixed with the aqueous phase prior to homogenization with the oil phase. Preferred agents include hydroxylated benzoate esters, such as methyl paraben, propyl paraben, 1-(3-chlorallyl)-3,4,7-triaza-1-azoniaadamantane chloride (DOWICIL, $(C_6H_{12}N_4(CH_2CHCHCl)Cl)$, butylated hydroxytoluene (BHT), and other antimicrobial agents known to those of skill in the art.

The oil-in-water emulsion is generally prepared by mixing the water and the surfactant along with any additional hydrophilic components, such as a fatty alcohol and a preservative, in a first container. The oil components, such as a triglyceride and a fatty glyceride, are mixed in a second container. The water phase is added to the oil phase for formation of the emulsion by agitation, such as by homogenization or emulsification, or by a micro-emulsion technique which does not involve agitation. The resulting emulsion preferably has water as the continuous phase and oil as the dispersed phase. The oil droplets in the dispersed oil phase preferably have sizes of less than about 1 μm, more preferably less than about 0.5 μm, in diameter. The droplet size, of course, is readily adjusted by mixing conditions, *e.g.*, shear and time of mixing, etc.

Typically, the liposomes include between 1-20% of the surfactant, more preferably between 2-15% (percentages are weight percentages based on the total lipid vesicle

composition, including the lipid phase described above). The lipophilic oil constitutes typically between 1-10% of the liposome composition. A fatty glyceride when added is typically between 0.1-5% of the composition and a fatty alcohol, when added, is typically between 0.1-5% of the composition.

C. Preparation of Biphasic Lipid Vesicles

Preparation of biphasic lipid vesicles has been described in detail, for example, in PCT Publication Nos. WO 95/03787 and in U.S. Patent No. 5,853,755. Briefly, the selected vesicle-forming lipid and the selected acylated amino acid are solubilized in a suitable solvent, which in a preferred embodiment, is a pharmaceutically acceptable hydrophilic solvent, such as a polyol, *e.g.*, propylene glycol, ethylene glycol, glycerol, or an alcohol, such as ethanol, or mixtures of such solvents. Depending on the physicochemical properties of the lipid components and on the selected solvent, it may be necessary to warm the mixture, for example, to between 40-80°C.

In a separate container, the surfactant-stabilized oil-in-water emulsion described above is prepared. A concentrated aqueous solution of interferon-α is also prepared, and biphasic lipid vesicles are formed by simultaneously mixing the oil-in-water emulsion and the concentrated drug solution with the solubilized lipids. The emulsion and the lipid components are mixed under conditions effective to form multilamellar vesicles having in the central compartment the oil-in-water emulsion.

It will be appreciated that the amount of a therapeutic agent entrapped in the lipid vesicles is readily controlled and varied by the concentration of the solution of therapeutic agent used during lipid vesicle formation.

The size of the vesicles is typically between about 0.1-100 μm. A lipid vesicle size of between about 0.5-5 μm is preferred, which can be most readily obtained by adjusting the mixing conditions.

Generally, the composition of lipid vesicles has a consistency similar to a cream without further addition of thickening or gelling agents, and, therefore, are readily applied directly to the skin of a subject for transdermal administration of the entrapped agent. Alternatively, the lipid vesicle composition can be readily incorporated into the reservoir of a transdermal device.

III. Transdermal Delivery

In studies performed in support of the invention, three model therapeutic agents were administered transdermally in combination with an acylated amino acid. The therapeutic agents were interferon-alpha (IFN-α), insulin, and albumin.

A. IFN-α

Biphasic lipid vesicles having entrapped IFN-α and an acylated amino acid were prepared for studies in support of the invention. These *in vitro* and *in vivo* studies will now be described.

1. *In vitro*

Biphasic lipid vesicles were prepared as described in Example 20A. The lipid vesicles were comprised of IFN-α and an acylated amino acid selected from PDM_1, PDM_3, PDM_4, PDM_5, PDM_{17}, PDM_{18}, PDM_{27} and MLL (see Tables 1A-1F for chemical name and structure). The composition of the biphasic lipid vesicles is set forth in Table 2.

Table 2
Biphasic lipid vesicle composition for *in vitro* study

LIPID PHASE Component (amount)[1]	OIL-IN-WATER EMULSION Water Phase Component (amount)	OIL-IN-WATER EMULSION Oil Phase Component (amount)
Phosphatidylcholine (5%)	Phospholipid EFA[2] (4%)	Olive oil (10%)
Acylated amino acid[3] (2%)	Methyl paraben (0.15%)	Glycerol monostearate (1%)
Propylene glycol (7%)	Propyl paraben (0.05%)	Synthetic beeswax (0.28%)
Cholesterol (2%)	Cetyl alcohol (0.6%)	
Stearic acid (1%)		

[1]amount reported as total weight percent

[2]linoleamidopropyl propylene glycol-dimonium chloride

[3]vesicles were prepared using the following acylated amino acids: PDM1, PDM3, PDM4, PDM5, PDM17, PDM18, PDM27 and MLL

The vesicles were prepared by simultaneously mixing the oil-in-water emulsion and an aqueous solution containing five million units of IFN-α with the solubilized lipid phase.

After vesicle preparation, a thin layer of the lipid vesicle suspension was observed under an optical microscope (Reichert Microstar IV) to measure the vesicle particle size and size homogeneity.

As a comparative control, a propylene glycol-based formulation was prepared by dissolving the same amounts of IFN-α and the selected acylated amino acid in propylene glycol.

The *in vitro* diffusion of IFN-α into human skin was determined using flow-through diffusion cells, as detailed in Example 20B. About 100 mg of each test formulation was applied on the stratum corneum side of the skin and allowed to remain for 24 hours. The amount of IFN-α that penetrated into the skin was assessed using a bioassay that measures interferon antiviral activity (Example 20C).

Table 3 shows the average amount of IFN-α in the skin at the end of the 24 hour test period, as determined by antiviral activity, for each of the test formulations.

Table 3

Acylated Amino Acid[1]	Average Amount of IFN-α in skin[2] (U/mg protein)		P values[3]
	Comparative formulation: Propylene glycol + Acylated Amino Acid	Lipid vesicles + acylated amino acid	
PDM1	539±197	290±172	>0.1
PDM3	583±347	2022±1046	<0.05
PDM4	868±348	1707±538	>0.1
PDM5	261±522	35±24	n.s.
PDM17	306±500	540±239	n.s.
PDM18	136±74	84±52	n.s.
PDM27	336±155	92±13	n.s.
MLL	---	250±112	---

[1]see Tables 1A-1F for compound name and structure

[2]n=4

[3]by unpaired t-test, comparison of lipid vesicle formulation and PG formulation

The data in Table 3 shows that incorporation of an acylated amino acid into lipid vesicles can increase the dermal and transdermal delivery of IFN-α. The data also shows that some acylated amino acids are particularly effective for dermal delivery of IFN-α, whereas other are more effective for transdermal delivery. For example, the acylated amino acids PDM_3 and PDM_4 when incorporated into lipid vesicles achieved a significant increase in delivery of IFN-α to the skin.

2. *In vivo*

a. Guinea Pigs

The transdermal absorption of IFN-α from formulations containing N-eicosanoyl-L-serine (PDM_3), N-eicosanoyl threonine (PDM_4) or monolauroyl lysine (MLL) entrapped in biphasic lipid vesicles was measured using guinea pigs as an *in vivo* model. The *in vivo* tests were conducted as described in Example 21. The lipid vesicles were prepared as described in Example 20A and were placed in the reservoir of a transdermal drug delivery device, similar to those described in U.S. Patent No. 5,718,914 (which is incorporated by reference in its entirety), except that the device contained no microporous membrane or other layer between the lipid vesicle formulation and the skin of the animal. The devices were placed on a section of shaved skin of each animal.

Fig. 1A is a bar graph showing the serum antiviral activity for animals treated with the three lipid vesicle test formulations and for control animals that where untreated. As seen, the formulation containing PDM_3 achieved a significant increase in transdermal skin penetration.

Fig. 1B is a bar graph showing the antiviral activity in skin homogenates prepared from the skin of guinea pigs after transdermal administration of the IFN-α lipid vesicle formulations. As seen, the animals treated with the lipid vesicles containing the acylated amino acids PDM_3 and PDM_4 show the highest antiviral activity at about 7,000 U/mg protein.

b. Humans

In other studies performed in support of the invention, dermal and transdermal delivery of IFN-α from biphasic lipid vesicles containing an acylated amino acid monolauroyl lysine (MLL) was evaluated. The lipid vesicles were prepared according to the process described in Example 20A with the composition set forth in Table 4.

Table 4
Composition of Biphasic Lipid Vesicles for Transdermal Administration of IFN-α to Human Subjects

LIPID PHASE	OIL-IN-WATER EMULSION	
Component (amount)[1]	Water Phase Component (amount)	Oil Phase Component (amount)
Phosphatidylcholine (10%)	Phospholipid EFA[2] (4%)	Canola oil (4%)
Monolauroyl lysine[3] (2%)	Methyl paraben (0.15%)	Glycerol monostearate (1%)
Propylene glycol (7%)%	Propyl paraben (0.05%)	Beeswax (0.28%)
Cholesterol (2%)	DOWICIL 200[4] (0.05%)	
	Cetyl alcohol (0.6%)	

[1]amount reported as volume percent
[2]linoleamidopropyl propylene glycol-dimonium chloride
[3]see Table 1 for structure
[4]Dowicil = 1-(3-chlorallyl)-3,4,7-triaza-1-azoniaadamantane chloride

The lipid vesicles were prepared with three dosages of IFN-α, 5 MU, 15 MU and 40 MU, by addition of an aqueous solution at the dosage concentration to the lipid phase during vesicle formation, as described in Example 22A.

The human subjects were divided randomly into three groups for treatment with a transdermal patch containing a lipid vesicle compositions at one of the three IFN-α concentrations. The experimental protocol is set forth in Example 22C. Briefly, in Phase I of the study, a placebo transdermal patch containing the biphasic lipid vesicles described in Table 4 and prepared as described in Examples 20A-20B with no IFN-α was adhered to the inner upper arm. After treatment with the placebo, skin biopsies and blood samples were collected from each subject. In Phase II of the study, each subject was treated with a transdermal patch containing IFN-α entrapped in biphasic lipid vesicles at one of the three concentrations: 5 MU/g formulation/patch, 15 MU/g formulation/patch or 40 MU/g formulation/patch. After treatment for 48 hours, skin biopsies and blood samples were taken and analyzed. The biopsy samples were analyzed by immunohistochemistry, antiviral assay, ELISA and 2-5A synthetase assays (see Example 20D-20F). The

blood samples were used to prepare serum for the antiviral and ELISA assays and to extract peripheral blood mononuclear cells for the 2-5A synthetase assay.

3. Dermal Drug Delivery: Analysis of Skin Biopsies

Skin biopsies analyzed by the antiviral assay for each subject are shown in Figs. 2A-2C. The antiviral assay demonstrates delivery of IFN-α by the presence of antiviral bioactivity due to IFN-α or by possible induction of other antiviral compounds. Table 5 summarizes the average antiviral activity in skin homogenates for each of the three treatment groups.

Table 5
Antiviral activity in skin homogenates of subjects treated with IFNα in a biphasic delivery transdermal patch for 48 hours

Formulation	Antiviral Activity[1] (U/mg protein in skin homogenate) mean ± SEM (range)		
	Topical dose applied		
	5 million Units	15 million Units	40 million Units
INF-α Formulation	120 ± 30 (n=5) (15 - 200)	380 ± 60(n=5) (200 - 500)	400 ± 80 (n=7) (160 - 930)
Control, placebo formulation	7 ± 2 (n=5)	13 ± 5 (n=5)	not determined
Untreated skin	not determined	not determined	75 ± 30 (n=7)
Statistics[2]	$p < 0.05$	$p < 0.005$	$p < 0.01$

[1]data are not corrected for loss of activity due to manufacturing and treatment.
[2]Statistical analysis by paired t-test.

The results in Table 5 from the antiviral assay indicate a dose dependent increase in antiviral activity. There was significant 5-fold average increase in delivery as the dose was increased from 5 to 15 MU. The expression of data per mg protein in skin homogenate accounts for skin biopsy thickness and recovery of tissue in the homogenization process.

Baseline levels of antiviral activity was determined in untreated volunteers (the 40 MU-dose group), as well as in volunteers treated with a placebo biphasic delivery system prior to treatment with the active formulations. Also as seen in Table 5, both untreated and placebo treated controls (baseline) showed relatively low levels of antiviral activity (overall range 7-75 U/mg protein in homogenate). The inter-individual variability of the

baseline was taken into account in the calculation of fold increase in antiviral activity in the skin post-treatment vs. pre-treatment, since each individual served as his/her own control.

Figs. 3A-3C show the IFN-α concentration in the skin homogenates for each individual in each of the three treatment groups. The ELISA assay demonstrates delivery of IFN-α by a sandwich immunoassay using an IFN-α-specific antibody and a horseradish peroxidase labelled secondary antibody for detection. The data show for each individual in Figs. 3A-3C is averaged for each treatment group in Table 6.

Table 6
Amount of IFN-α detected by ELISA in skin homogenates of volunteers treated topically with IFN-α in a biphasic delivery system for 48 hours

Formulation	Amount of IFN-α[1] (pg/mg protein in skin homogenate) mean ± SEM (range)		
	Topical dose applied		
	5 million Units	15 million Units	40 million Units
INF-α Formulation	40.1 ± 12.8 (n=10) (20.6 - 90.1)	122.4 ± 25.9 (n=10) (55.5-186.0)	107.5 ± 18.1 (n=7) (55.2 - 186.3)
Control Placebo formulation	1.6 ± 1.0 (n=5)	3.2 ± 2.0 (n=5)	not determined
Untreated skin	not determined	not determined	not determined
Statistics[2]	$p < 0.05$	$p < 0.02$	$p < 0.002$

[1]data are not corrected for loss of activity due to manufacturing and treatment.
[2]Statistical analysis by paired t-test

The data in Table 6 based on the ELISA assay shows a similar trend as observed for the data analyzed by the antiviral assay (Table 5). Namely, there was a 3-fold increase in IFN-α levels in the post-treatment skin homogenates of subjects treated with a 15 MU dose of IFN-α compared to the subjects treated with a 5 MU dose of IFN-α. No significant further increase was observed as the dose was increased to 40 MU. Baseline IFN-α levels were very low or undetectable under the conditions used in this study, as can be seen from the placebo and untreated control values in the table.

4. Transdermal Drug Delivery: Analysis of Blood Samples

The blood samples collected from each subject before and after treatment with transdermal patch containing a biphasic lipid vesicle composition with entrapped IFN-α

were analyzed by the antiviral assay to determine the amount of antiviral activity in the serum of each volunteer. The results for each subject are shown in Figs. 4A-4C. The results show a significant increase in serum antiviral activity in the individual subjects in each treatment group.

Induction of 2-5A synthetase enzyme activity in peripheral blood mononuclear cells (PBMC) was used as an indication for the systemic delivery of IFN-α from biphasic delivery systems. The 2-5A synthetase enzyme activity for each individual in each treatment group is shown in Figs. 5A-5C. The averaged data within each treatment group is summarized in Table 7.

Table 7

2-5A synthetase enzyme activity in PBMC cellular fraction of blood taken from subjects treated transdermally with IFN-α in a biphasic delivery system for 48 hours

Formulations	2-5A synthetase[1] (pmole 2-5A polyadenylate/mg protein/h) mean ± SEM (range)		
	Topical dose applied		
	5 million Units	15 million Units	40 million Units
INF-α Formulation	222.3 ± 41.8 (n=5) (108.6 - 311.0)	213.7 ± 42.5 (n=5) (107.0 - 323.3)	1639 ± 421 (n=4) (232.9 - 2660)
Untreated skin control	48.5 ± 18.3 (n=5)	110.7 ± 34.5 (n=5)	330.9 ± 182.9 (n=4)
Placebo control	35.4 ± 11.4 (n=5)	35.4 ± 11.4 (n=5)	not done
Statistics[2]	$p < 0.02$	$p < 0.02$	$p < 0.10$

[1]data are not corrected for loss of activity due to manufacturing and treatment

[2]Statistical analysis by paired t-test

Each individual served as his/her own control to account for inter-individual variability. As seen in the individual data shown in Figs. 5A-5C. overall baseline levels varied from about 50-300 pmol/mg protein/h. There was a significant induction in enzyme activity in all three treatment groups compared to their untreated controls ($p<0.05$). There was no significant difference between the treatment groups receiving 5 MU IFN-α and 15 MU IFN-α, however, the difference between the groups treated with 15 MU IFN-α and 40 MU IFN-α was significant at $p<0.05$.

The skin biopsy taken from the site of application of the biphasic lipid vesicle-containing transdermal patch of each test subject was processed for

immunohistochemistry. A skin sample from a representative individual in the treatment group receiving 40 MU IFN-α was compared visually to a skin section of control, untreated skin after staining both samples with anti-IFN-α antibodies. The photomicrographic images, while not shown here, show that the skin site treated with the biphasic delivery system has IFN-α specific immunostaining throughout the stratum corneum, epidermis and dermis. This staining pattern was characteristic in all volunteers in this treatment group.

Skin sections were also stained for Ki67 nuclear protein in proliferating cells using anti-M1B1 monoclonal antibodies (Rose *et al.*, *J. Clin. Pathol.*, 47:1010 (1994)) and visualized. The photomicrographs of the skin sections are not shown here, but the anti-proliferative effect of IFN-α in the treated skin is evident from the decreased staining in the skin treated with IFN-α.

Table 8 shows the results of an anti-proliferation assay performed on skin sections of the test subjects in each treatment group after treatment with the respective dosages of IFN-α for 48 hours. The number of stained cells were counted before and after treatment sections. The decreased number of stained cells were expressed as a percentage.

Table 8
Anti-proliferation assay on skin sections of human volunteers after topical IFN-α treatment

Dose (MU)	Average % decrease of proliferative cells stained by anti-Ki67 antibody[c]	anti-proliferative ratio[c]	p Value[d]
5[a]	14	1.21	n.s.
15[a]	10	1.32	n.s.
40[b]	38	2	< 0.1

[a]compared to placebo treated skin

[b]compared to untreated skin

[c]average number of proliferating cells in untreated skin (or placebo treated)/ average number of proliferating cells after treatment

[d]statistical analysis by paired t-test

The *in vivo* results from the human subjects indicate that the biphasic lipid vesicle composition of the invention when administered transdermally is effective to deliver

therapeutically significant amounts of IFN-α to the skin and the blood.

B. Transdermal Administration of Insulin

A method for the long term introduction of insulin into the body in a non-invasive manner is desirable, as a typical insulin-dependent patient with diabetes patient may live with the disease for more than 40 years, over which time 60;000 to 70,000 insulin injections are given. Approximately 5% of the North American population suffer from diabetes resulting in enormous medical costs, and in the U.S. in 1995 the cost of diabetes, both direct medical cost and indirect costs, was approximately $95 billion. The major problem with transdermal delivery of insulin is the inability of the protein to normally cross the stratum corneum. Recombinant human insulin has a low solublility at physiological pH and frequently forms high molecular mass hydrophilic hexamers. This hexamer (36 kDa) would be unlikely to penetrate the hydrophobic stratum corneum in any significant concentrations without the active assistance of a delivery system. Accordingly, in one embodiment, the invention provides for non-invasive delivery of insulin.

Biphasic lipid vesicles having entrapped insulin and an acylated amino acid were prepared for *in vivo* studies in support of the invention, now to be described.

Compositions for transdermal administration of insulin were prepared as described in Example 23. Biphasic lipid vesicles having the composition set forth in Table 9 were prepared.

Table 9
Composition of Biphasic Lipid Vesicles for Transdermal Administration of insulin to Animals

LIPID PHASE	OIL-IN-WATER EMULSION	
	Water Phase	Oil Phase
Component (amount)[1]	Component (amount)	Component (amount)
Phosphatidylcholine (14%)	Phospholipid EFA[2] (0.5%)	Olive oil (0.25%)
Acylated amino acid[3]: PDM_{27}, PDM_1, PDM_4 or PDM_{17} (1%)	Methyl paraben (0.04%)	
Propylene glycol (13%)	Propyl paraben (0.01%)	
Cholesterol (4%)		

[1]amount reported as weight percent
[2]linoleamidopropyl propylene glycol-dimonium chloride
[3]see Table 1 for chemical names and structures

The vesicles were prepared by simultaneously mixing the oil-in-water emulsion and an aqueous solution with the solubilized lipid phase. The resulting biphasic lipid vesicles formulations had 50 mg porcine insulin/g formulation. One additional formulation was prepared with human insulin at 10 mg insulin/g formulation and with the acylated amino acid PDM_{27}. The biphasic lipid vesicle formulations, which differ only in the acylated amino acid and in the type/concentration of insulin are summarized in Table 10.

Table 10
Summary of Biphasic Lipid Vesicles for *in vivo* studies

Formulation Identification[1]	Acylated Amino Acid	Insulin type and concentration
"PDM_{27}/10mg"	PDM_{27}	Human, 10 gm insulin/g formulation
"PDM_{27}/50mg"	PDM_{27}	Porcine, 50 mg insulin/g formulation
"PDM_1"	PDM_1	Porcine, 50 mg insulin/g formulation
"PDM_4"	PDM_4	Porcine, 50 mg insulin/g formulation
"PDM_{17}"	PDM_5	Porcine, 50 mg insulin/g formulation

[1]all biphasic lipid vesicles had the formulation set forth in Table 9 with the indicated acylated amino acid and insulin concentration indicated here.

For the *in vivo* evaluation of the biphasic lipid vesicle formulations, an insulin dependent diabetes mellitus (IDDM) animal model in rats was used. As described in Example 24, a diabetic state was induced in rats by injection of streptozotocin, which destroys pancreatic β-cells. Normal rat blood glucose ranges from approximately 3-7 mM during the day, with the level higher just after feeding. Streptozotocin-induced diabetic rats having a higher blood glucose, typically greater than 8mM. A blood glucose level of between 10-16 mM is considered a mild diabetic state, and a blood glucose of greater than 20 mM being more severe.

A control group of rats were each injected with citrate buffer. Three days after the streptozoticin or citrate buffer injections, the rats were divided into groups for treatment:

(1) control, healthy rats (no streptozoticin, no treatment);

(2) control, streptozoticin-induced diabetes, no treatment;

(3) streptozoticin-induced diabetes + subcutaneous injection (1 mg insulin; 28 U);

(4) streptozoticin-induced diabetes + transdermal formulation "PDM_{27}/50mg" (see Table 10); and

(5) streptozoticin-induced diabetes + transdermal formulation "PDM_{27}/10mg" (see Table 10).

The animals receiving insulin subcutaneously received one injection at the beginning of the test. Blood samples were taken at intervals for the following 12 hours to track the serum insulin and blood glucose levels. Fig. 6 is a plot showing the insulin concentration in the serum following subcutaneous injection of insulin (1 mg) to a diabetic rat that is characteristic of the treatment group. The mean duration of response for the animals in the group treated with subcutaneous insulin was 3.7 ± 0.3 hrs (n=29). Peak subcutaneous insulin effects reduced the blood glucose to 3.8 ± 0.3 mmol/L (n=29).

Animals in the transdermal test groups received a transdermal device containing a test formulation placed on the skin at the start of the test period. The device was left in place for 2 days, during which time the blood was drawn periodically to analyze blood glucose levels. After the treatment period, skin samples were taken and analyzed for quantity of insulin, and blood was collected and the amount of insulin in the plasma was determined.

Fig. 7 shows blood glucose levels, in mmol/L, as a function of time, in rats treated with transdermally administered insulin from biphasic lipid vesicles containing the acylated amino acid PDM_{27} ("PDM_{27}/50mg" (see Table 10); open triangles). The transdermal device contained 200 mg of 50 mg insulin/g formulation. The blood glucose levels of non-

diabetic, healthy, untreated rats (solid triangles) and of diabetic rats left untreated (open circles) are also shown.

As can be seen in Fig. 7, the blood glucose levels of untreated, diabetic rats (open circles) fluctuated with feeding over the test period. The fluctuation in blood glucose level for the untreated diabetic rats was more pronounced than for the normal, non-diabetic rats (solid triangles), confirming induction of a diabetic state in the streptozotocin-injected animals. In the rats treated with the biphasic lipid vesicles containing insulin (open triangles), the blood glucose levels fluctuated less compared to the untreated diabetic rats, with the blood glucose level dropping to a "normal" (approximately 3-7 mm) range in the 24-30 hour time frame.

Fig. 8 shows the change in blood glucose levels, in mmol/L, as a function of time, in rats treated with a transdermal biphasic lipid vesicle composition containing the acylated amino acid PDM_{27} ("PDM_{27}/10mg" (see Table 10): open triangles). The transdermal device contained 1000 mg of a 10 mg insulin/g formulation. The blood glucose levels of diabetic rats treated with a subcutaneous injection of insulin (open circles) and of normal, healthy rats (solid triangles) are also shown.

Fig. 8 shows that transdermal administration of insulin from the biphasic lipid vesicles effectively reduced the blood glucose levels over the 60 hour test period. The animals treated subcutaneously with insulin (open circles) experienced a reduction in blood glucose for a period of less than 10 hours.

The data in Figs. 7 and 8 also show the effect of the insulin concentration in the lipid vesicle formulation on blood glucose levels. A 200 mg amount of vesicles containing 50 mg insulin/g formulation resulted in a shorter (6-20 hours), more intense therapeutic response, returning the blood glucose levels to normal baseline (Fig. 7), as compared to a 1000 mg amount of vesicles containing 10 mg insulin/g formulation, which provided a more extended, less intense response.

Figs. 7 and 8 also show that the composition containing a 200 mg amount of vesicles containing 50 mg insulin/g formulation had a lag time of about 16 hours (Fig. 7). The composition containing 1000 mg of vesicles containing 10 mg insulin/g formulation had a lag time of about 4 hours (Fig. 8).

In another study, the duration of the response to transdermally administered insulin from biphasic lipid vesicles was compared to the duration of the response to subcutaneously administered insulin in solution and entrapped in biphasic lipid vesicles. As shown in Fig.

9. the mean response duration for subcutaneously administered insulin at a does of 28 U in solution form was about 3.8 hours. Insulin at a dose of 280 U was administered from biphasic lipid vesicles transdermally (dotted bars) and subcutaneously (lined bars). Herein the mean response duration for subcutaneously injected biphasic lipid vesicles containing insulin was about 40 hours. The mean response duration for topically-applied biphasic lipid vesicles containing insulin was about 44 hours. Thus. topical administered of the biphasic lipid vesicles formulation containing insulin achieved the same prolonged response as subcutaneously administered biphasic lipid vesicles. without requiring an injection.

As noted in Example 23. the pH of the biphasic lipid vesicle formulations was adjusted with 1 M NaOH to between 4.5-5.5. Other studies (not reported here) with biphasic lipid vesicle formulations having a pH below about 3.5 resulted in little transdermal administration of insulin. Accordingly, in one embodiment of the invention. the pH of the biphasic lipid vesicle formulation is greater than 3.5. more preferably the pH of the biphasic lipid vesicle formulation is between 3.5-7.5, and still more preferably the pH is between 3.5-5.5.

In another study performed in support of the invention. diabetic animals were treated transdermally with the formulations set forth in Table 10 ("PDM_1". "PDM_2" "$PDM_{1'}$"). The animals were prepped and the patches adhered as set forth in Example 24. The patches were left in place for three days after which blood and skin samples from the animals were collected for analysis (Example 24). Table 11 shows the amount of insulin in the serum of the test animals as well as the amount of insulin in the skin after treatment.

Table 11
Amount of Insulin in the serum and in the skin after transdermal administration for three days.

Treatment	Acylated Amino Acid[1]	Serum Insulin (pg/mL)	Dermal Skin Insulin (pg/mL homogenate)
Control	--	10.6 ± 6.7	77 ± 43
control, diabetic rats untreated	--	7.6 ± 3.3	41 ± 25
Subcutaneous insulin	--	750 ± 370	31 ± 26
Transdermal formulation "PDM_1"	PDM1	35 ± 8	4746 ± 1189
Transdermal formulation "PDM_{27}/50mg"	PDM27	6860 ± 1930	1255 ± 331
Transdermal formulation "PDM_4"	PDM4	115 ± 29	3876 ± 1442
Transdermal formulation "PDM_{17}"	PDM17	not determined	2515 ± 1542

[1]see Tables 1A-1F for chemical name of structure of acylated amino acid

The overall efficiency of the transdermally administered insulin was determined by taking the subcutaneously administered route as being 100% efficient, where in one study, subcutaneous administration of insulin decreased the blood glucose 17.2 mmol/L. In the same study, a transdermal liposome formulation with PDM_{27} as the acylated amino acid decreased the blood glucose 16.2 mmol/L. The percent efficiency of the transdermal formulation is then (16.2/17.2)x100, or 94.2%. To reach a therapeutic blood glucose value of 8 mmol/L or less from a diabetic blood gluose level of 21 mmol/L, a decrease of 13 mmol/L is needed, or a percent efficiency of 75.6% ((13/17.2)X100).

Table 12 summarizes the mean blood glucose levels at the 24 hour time point and the average response duration for several of the animals in the control group, in the diabetic, untreated group, in the subcutaneously-treated group, and in the group treated transdermally with biphasic lipid vesicles containing PDM_{27} and insulin at a dose of 10mg insulin/g formulation.

Table 12

Blood Glucose level and time to response after 24 hours of transdermal insulin administration

Insulin Treatment	Blood Glucose ± SEM (mmol/L)	Range (mmol/L)	Response Duration ± SEM (hours)	Range (hrs)
None (n = 19)	4.67 ± 0.13	3.3 - 5.8	N/A	N/A
Diabetic, untreated (n = 31)	22.58 ± 0.59	14.9 - 26.6	N/A	N/A
Subcutaneous Injection (1 mg) (n = 29)	3.84 ± 0.28	1.6 - 7.9	3.69 ± 0.31	1 - 10
Transdermal Formulation "PDM_{27}/10mg" (n = 31)	10.00 ± 0.69	1.8 - 15.9	18.78 ± 2.2	5 - 49

The depth of penetration into the skin after transdermal administration of the transdermal, biphasic lipid vesicle formulations was determined using confocal microscopy (Example 24). Photomicrographs, not shown here, were taken from skin sections of diabetic rats treated with the transdermal biphasic lipid vesicle compositions containing insulin and an acylated amino acid. In the images of the skin treated with the liposome formulation containing PDM_{27}, the fluorescent distribution throughout the epidermis and dermis was evident, indicating liposome penetration to these depths. Skin treated with the formulations containing PDM_1 and PDM_3 showed that the insulin is superficially in the stratum corneum upper epidermis or around the hair shaft and follicles.

The information from the microscopy and the *in vivo* studies show that transdermal delivery of insulin was significantly enhanced using the liposome formulation containing acylated amino acid PDM_{27}. Formulations containing acylated amino acid PDM_1, PDM_3 and PDM_{17} resulted in significant dermal delivery without significant delivery into the blood. Thus, it will be appreciated that the compositions of the invention can be optimized to achieve primarily transdermal or primarily dermal delivery as desired.

3. *In vitro* Administration of Albumin

The *in vitro* diffusion of albumin (bovine, MW 60kDA) through skin from several albumin-containing composition was measured. The test composition in accord with the invention was comprised of the acylated amino acid monolauroyl lysine (MLL) and albumin

entrapped in liposomes having an oil-in-water emulsion in the core compartment, prepared as described in Example 20A. Comparative compositions were (1) albumin entrapped in conventional, aqueous core liposomes, with no acylated amino acid and (2) an aqueous solution of albumin. The albumin, which as fluorescently labeled, was present in the test compositions at a concentration of 100 μg/g.

The test formulations were applied to the stratum corneum side of skin mounted in flow-through diffusion cells. Twenty-four hours after application, the skin was cleansed and sectioned into thicknesses of about 0.5 mm. The sections were scanned at 10μm depth with a Multiprobe 2001 CLSM with a Nikon Diaphot cross-epifluorescence inverted microscope as described in Example 24B.

In the image for the control skin, not shown here, no fluorescence is detected. Nor was fluorescence detected in the images corresponding to skin treated with the aqueous solution of albumin and with the conventional, aqueous-core liposomes. The skin treated with the oil-in-water biphasic lipid vesicles containing MLL show fluorescence in the epidermis and dermis, indicative of transdermal administration of albumin.

IV. Methods of the Invention

In another aspect, the invention includes a method for selecting a composition for therapeutic transdermal administration of a macromolecule. The method includes selecting from a library of acylated amino acids (such as those in Tables 1A-1F), one or more acylated amino acids for analysis. Next, a carrier vehicle is selected, where the carrier vehicle can be any of those described above, and in particular is selected from an aqueous-based solution, a non-aqueous solution and a suspension of liposomes. The acylated amino acid is admixed with the carrier vehicle and the macromolecule or therapeutic agent is also added. The transdermal skin penetration of the therapeutic agent is determined, either by *in vitro* or *in vivo* methods.. For example, the *in vitro* penetration method used above to determine penetration of albumin into the skin via microscopy can be employed.

Depending on the number of acylated amino acids to be screened, these steps are repeated as needed to determine the skin penetration of the macromolecule in each of the test compositions.

An acylated amino acid and carrier vehicle combination suitable to achieve a transdermal penetration rate sufficient for therapy is selected as the composition for

therapeutic transdermal administration.

V. Examples

The following examples further illustrate the invention described herein and are in no way intended to limit the scope of the invention.

EXAMPLE 1

Synthesis of N-Caprylovl-L-threonine methyl ester (PDM-1)

A. Preparation of caprylic acid N-hydroxysuccinimide ester (Intermediate)

Caprylic acid (7.2 g, 50 mmol) was dissolved in 40 mL of dichloromethane and 10 mL of N,N-dimethylformamide. 1-N -Hydroxysuccinimide (5.75 g , 50 mmol) and N,N-dicyclohexylcarbodiimide (50 mmol, 100 mL, of 0.5 M solution) was added at -50°C and the solution allowed to warm up to room temperature and the reaction mixture stirred at room temperature for 16 hr. Dicyclohexyl urea precipitate was filtered off. The capryloyl NHS ester was purified by crystallization from diethyl ether- n-hexane solution (9.8 g, 82%).

B. Preparation of N-Caprylovl-L-threonine methyl ester

L-threonine methyl ester was added to 1 equivalent of caprylic acid N-hydroxysuccinimide ester in DCM 20 mL and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The product was extracted with ethyl acetate, washed with brine and the organic phase dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate and n-hexane. The product was filtered and dried under vacuum.

EXAMPLE 2

Synthesis of N-Eicosanoyl-L-serine (PDM-3)

A. Preparation of Eicosanoic acid N-hydroxysuccinimide ester Intermediate

Eicosanoic acid (5.0 mmol) was dissolved in 40 mL of dichloromethane and 10 mL of N,N-dimethylformamide. 1-N -Hydroxysuccinimide (5.0 mmol) and N,N-dicyclohexyl-carbodiimide (5.0 mmol 10.0 mL, of 0.5 M solution) was added at -5°C and the solution allowed to warm up to room temperature and the reaction mixture stirred

at room temperature for 16 hr. Dicyclohexyl urea precipitate was filtered off. The filtrate was concentrated and the N-hydroxysuccinimide ester derivative purified by crystallization from dichloromethane-n-hexane solution.

B. Preparation of N-Eicosanoyl-L-serine

A solution of L-serine (1.2 mmol) and sodium bicarbonate (1.2 mmol) in water, 2 mL was added to N-hydroxysuccinimidyl eicosanoate dissolved in p-dioxane:tetrahydrofuran (1:1), 20 mL, and stirred at room temperature for 10 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was evaporated on a rotary evaporator under vacuum. The residual aqueous phase was cooled in an ice bath and acidified with concentrated hydrochloric acid to pH 3.0. The product was filtered off and crystallized from dichloromethane - ethyl acetate solution.

EXAMPLE 3

Synthesis of N-Eicosanoyl-L-threonine (PDM-4)

A solution of L-threonine (1.2 mmol) and sodium bicarbonate (1.2 mmol) in water, 2 mL was added to N-hydroxysuccinimidyl eicosanoate dissolved in p-dioxane:tetrahydrofuran (1:1), 20 mL, and stirred at room temperature for 10 hr. The reaction mixture was treated with water for 1hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was evaporated on a rotary evaporator under vacuum. The residual aqueous phase was cooled in an ice bath and acidified with concentrated hydrochloric acid to pH 3.0. The product was filtered off and crystallized from dichloromethane - ethyl acetate solution.

EXAMPLE 4

Synthesis of Nα-Caprvloyl-ε-lauroyl-L-lysine methyl ester (PDM 5)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-Lauroyl-L-lysine methyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-

hydroxysuccinimide ester. The organic phase was washed sequentially with 1N hydrochloric acid, saturated sodium bicarbonate, and brine. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate- n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 5

Synthesis of N-α-Palmitoyl-ε-lauroyl-L-lysine methyl ester (PDM 17)

A. Preparation of Palmitic acid N-hydroxysuccinimide ester (PDM 21) (Intermediate)

To palmitic acid 2.560 g in dichloromethane was added N-hydroxysuccinimide 1.15 g, and dicyclohexylcarbodiimide (20 mL of 0.5M solution in DCC). The reaction mixture was stirred at room temperature for 12 hr and the dicyclohexyl urea filtered off. The product was crystallized from ethyl acetate and hexane to give 2.55 g (68% yield 1st crop) of the product.

B. Preparation of N-α-Palmitoyl-ε-lauroyl-L-lysine methyl ester

N-ε-Lauroyl-L-lysine methyl ester was added to 1.1 equivalents of palmitic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with 1N hydrochloric acid, saturated sodium bicarbonate, and brine. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate - n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 6

Synthesis of N-α-Lauroyl-ε-lauroyl-L-lysine methyl ester (PDM 18)

A. Preparation of Lauric acid N-hydroxysuccinimide ester PDM-22 (Intermediate)

To lauric acid 2.0 g in dichloromethane was added N-hydroxysuccinimide 1.15 g, and dicyclohexylcarbodiimide (20 mL of 0.5M solution in DCC). The reaction mixture was stirred at room temperature for 12 hr and the dicyclohexyl urea filtered off. The

product was crystallized from dichloromethane -n- hexane solution to give 2.5 g (79 yield 1st crop) of the product.

B. Preparation of N-α-Lauroyl-ε-lauroyl-L-lysine methyl ester

N-ε-Lauroyl-L-lysine methyl ester was added to 1.1 equivalents of lauric acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with 1N hydrochloric acid, saturated sodium bicarbonate, and brine. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate - n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 7

Synthesis of N-ε-Lauroyl-L-lysine ethyl ester (PDM-19)

N-ε-lauroyl-L-lysine (10 mmol) was suspended as a slurry in 20 mL of anhydrous ethanol. Thionyl chloride (12 mmol) was slowly added at 0°C and stirred for 30 minutes. The resulting solution was stirred at room temperature for 16 hrs. The excess ethanol was evaporated in vacuo on a rotary evaporator. Dichloromethane was added to the residue and aqueous saturated sodium bicarbonate solution. The precipitated material was filtered off. The organic phase was separated from the filtrate and dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The product was crystallized from dichloromethane-n- hexane solution.

EXAMPLE 8

Synthesis of N-ε-Lauroyl-L-lysine methyl ester (PDM-20)

N-ε-Lauroyl -L-lysine (10 mmol) was suspended as a slurry in 20 mL of anhydrous methanol. Thionyl chloride (12 mmol) was slowly added at 0°C and stirred for 30 minutes. The resulting solution was stirred at room temperature for 16 hr. The excess ethanol was evaporated under vacuum on a rotary evaporator. Dichloromethane was added to the residue and the product was neutralized with aqueous saturated sodium bicarbonate solution. The precipitated material was filtered off. The organic phase was

separated from the filtrate and dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The product was crystallized from dichloromethane-n-hexane solution.

EXAMPLE 9

Synthesis of N-α-Caprylovl-ε-lauroyl-L-lysine ethyl ester (PDM 27)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-Lauroyl-L-lysine ethyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with 1N hydrochloric acid, saturated sodium bicarbonate, and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 10

Synthesis of N-α-Maleoyl-ε-lauroyl-L-lysine ethyl ester (PDM 29)

N-ε-Lauroyl-L-lysine ethyl ester (5 mmol) was dissolved in dichloromethane. 2 equivalents of maleic anhydride dissolved in 1 mL dimethylformamide was added and stirred at room temperature for 3 hr. Sodium carbonate (5 mmol) and 200 L of water were added and stirred for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted anhydride. The product was acidified to pH 2.5 with concentrated hydrochloric acid at 0°C. The organic phase was washed sequentially with 1N hydrochloric acid, water and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 11

Synthesis of N-ε-Lauroyl-L-lysine n-propyl ester (PDM-41)

N-ε-Lauroyl-L-lysine was suspended as a slurry in n-propanol. Thionyl chloride, 2 equivalents, was slowly added at 0°C and stirred for 30 minutes. The resulting solution was stirred at room temperature for 16 hr. The excess propanol was evaporated on a rotary evaporator. Dichloromethane was added to the residue and the product was neutralized with aqueous saturated sodium bicarbonate solution. The precipitated material was filtered off. The organic phase was separated from the filtrate and dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The product was crystallized from dichloromethane-hexane solution.

EXAMPLE 12

Synthesis of N-ε-Lauroyl-L-lysine n-butyl ester (PDM-42)

N-ε-Lauroyl-L-lysine was suspended as a slurry in n-butanol. Thionyl chloride, 2 equivalents, was slowly added at room temperature and then heated at 60°C for 4 hr. The resulting solution was allowed to cool to room temperature and diethyl ether was added to precipitate the product. The product was cooled on ice and the precipitate filtered. Dichloromethane was added to the precipitate and the product was neutralized with aqueous saturated sodium bicarbonate solution. The precipitated material was filtered off. The organic phase was separated from the filtrate and dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The product was crystallized from dichloromethane-hexane solution.

EXAMPLE 13

Synthesis of N-ε-Lauroyl-L-lysine n-isoamyl ester (PDM-43)

(Mixture of isomers)

N-ε-Lauroyl -L-lysine was suspended as a slurry in isoamyl alcohol. Thionyl chloride, 2 equivalents, was slowly added at room temperature and then heated at 70°C for 4 hr. The resulting solution was allowed to cool to room temperature. Dichloromethane was added to the product and aqueous saturated sodium bicarbonate solution added The precipitated material was filtered off. The organic phase was separated from the filtrate and dried with anhydrous sodium sulfate, filtered, and

concentrated under vacuum. The product was crystallized from dichloromethane-hexane solution.

EXAMPLE 14

Synthesis of N-ε-Lauroyl-L-lysine n-dodecyl ester (PDM-45)

N-ε-Lauroyl -L-lysine was suspended as a slurry in n-dodecanol. Thionyl chloride, 2 equivalents, was slowly added at room temperature and then heated at 70°C for 3 hr. The resulting solution was allowed to cool to room temperature and diethyl ether was added to precipitate the product. The product was cooled on ice and the precipitate filtered. Dichloromethane was added to the precipitate and aqueous saturated sodium bicarbonate solution added. The precipitated material was filtered off. The organic phase was separated from the filtrate, dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The product was crystallized from dichloromethane-hexane solution.

EXAMPLE 15

Synthesis of N-α-Caprvloyl-ε-lauroyl-L-lysine propyl ester (PDM 46)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-Lauroyl-L-lysine n-propyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with saturated sodium bicarbonate, 1N hydrochloric acid, and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 16

Synthesis of N-α-Caprvloyl-ε-lauroyl-L-lysine n-butyl ester (PDM 47)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-Lauroyl-L-lysine n-butyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with saturated sodium bicarbonate, 1N hydrochloric acid, and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 17

Synthesis of N-α-Caprvloyl-ε-lauroyl-L-lysine isoamyl ester (PDM 48/49)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-lauroyl-L-lysine isoamyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with saturated sodium bicarbonate, 1N hydrochloric acid, and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 18

Synthesis of N-α-Caprvloyl-ε-lauroyl-L-lysine n-dodecyl ester (PDM 50)

Caprylic acid N-hydroxysuccinimide ester was prepared as described in Example 1A.

N-ε-lauroyl-L-lysine n-dodecyl ester was added to 1.1 equivalents of caprylic acid N-hydroxysuccinimide ester in dichloromethane and stirred at room temperature for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted N-hydroxysuccinimide ester. The organic phase was washed sequentially with saturated sodium bicarbonate, 1N hydrochloric acid, and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The

product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 19

Synthesis of N-α-Maleoyl-ε-lauroyl-L-lysine n-dodecyl ester (PDM 51)

N-ε-Lauroyl-L-lysine dodecyl ester (5 mmol) was dissolved in dichloromethane. 2 equivalents of maleic anhydride dissolved in 1 mL dimethylformamide was added and stirred at room temperature for 3 hr. Sodium carbonate (5 mmol) and 200 L of water were added and stirred for 12 hr. The reaction mixture was treated with water for 1 hr to hydrolyze the unreacted anhydride. The product was acidified to pH 2.5 with concentrated hydrochloric acid at 0°C The organic phase was washed sequentially with 1N hydrochloric acid, water and brine solution. The organic phase was separated and dried with anhydrous sodium sulfate, filtered and concentrated. The product was crystallized from ethyl acetate-n-hexane solution. The product was filtered and dried under vacuum.

EXAMPLE 20

In vitro Adminstration of IFN-α

Materials

IFN-α_{2b} (Intron A) was obtained from Schering-Plough. Synthetic beeswax was obtained from Croda, Toronto, Ontario. DOWICIL was obtained from Dow Chemical (Midland, Michigan). Phospholipid EFA™ was purchased from PEBRO (Missassauga, Ontario, Canada). Canola oil was obtained from Natural Oils International, Arleta California.

A. Biphasic Lipid Vesicle Preparation

An anhydrous lipid gel was prepared by mixing the following components together:

Component	Amount (% w/w)
Phosphatidylcholine[1]	5
Cholesterol	2
Acylated amino acid[2]	2
Stearic acid	1
Propylene glycol	7

[1]Phospholipon®90H, Rhone Poulenc Rorer American Lecithin Company, Dunbury CT
[2]see specific studies for the acylated amino acid added

The lipids and the solvents were weighted into a glass container and warmed to 65-75°C by intermittent heating, and gently mixed.

An oil-in-water emulsion was prepared by combining the hydrophilic ingredients in a container and combining the lipophilic ingredients in another container:

Hydrophilic Ingredients	Amount (% w/w)
distilled water	q.s. to 100
PEFA[1]	4.0
Methylparaben	0.15
Propylparaben	0.05
DOWICIL[2]	0.05
Lipophilic Ingredients	
canola oil	4.0
glyceryl monostearate	1.0
ceryl alcohol	0.6
synthetic beeswax	0.28

[1]PEFA = linolcamidopropyl propylene glycol-dimonium chloride
[2]Dowicil = 1-(3-chlorallyl)-3,4,7-triaza-1-azoniaadamantane chloride

The oil-in-water emulsion was prepared by adding the lipophilic mixture to the hydrophilic ingredient mixture at 60-80°C in a homogenizer at 20-80 psig for 5-30 minutes to obtain a small droplet size of less than about 0.5 μm.

Biphasic lipid vesicles were prepared by simultaneously adding the oil-in-water emulsion and an aqueous solution containing 5 million units of IFN-α to the lipid gel, which was warmed to 55°C. The gel, IFN-α and emulsion were vigorously mixed by vortexing or propeller mixing to achieve the desired particle size.

B. Diffusion Cell Studies

The diffusion of IFN-α into human skin was investigated using flow-through diffusion cells. Full thickness human skin (thickness approx. 2.0 mm) obtained from plastic surgery and kept at -20°C was placed in the flow through diffusion cells. The diffusion cells are designed such that fluid may be continuously pumped through them in order to maintain sink conditions (flow rate: 3 mL/hour). A phosphate buffer (PBS: 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 141.2 mM NaCl) isotonic with body fluids was used and the temperature was maintained at 32°C by a circulating water bath.

About 100 mg of formulation was applied on the stratum corneum side of the skin to 4 diffusion cells (n=4). Each experiment was conducted for a period of 24 hours continuously. After 24 hours, the skin was removed from the diffusion cell and the surface was washed with sterile distilled water (3 x 10 mL). Using an adhesive tape the residual formulation from the surface of the skin that was not removed in the washing process was stripped (1x).

The amount of IFN-α that penetrated into the skin was assessed using a bioassay that measures interferon antiviral activity. A skin homogenate was prepared using the skins. The IFN-α present in the homogenate was extracted by centrifugation with 2 mL of PBS. The accuracy of the antiviral activity bioassay was verified by spiking 100 U/ml IFN-α on an untreated skin homogenate supernatant and extracting the IFN-α via centrifugation. The supernatant was either PBS or PBS containing 0.005% of Tween 80. To check the possibility of interference from background IFN (interferon already present in the skin homogenate) the supernatant from skin homogenate where no interferon was added was also analyzed.

C. Antiviral Assay

Antiviral activities in skin homogenates were determined in triplicates of serial dilutions using Madin-Darby bovine kidney (MDBK) cells and vesicular somatitis virus (VSV) in 96-well microtiter plates. Activity (U) in the samples was calculated based on serial dilutions of an IFN-α standard.

The MDBK cells were grown to confluency in 150 cm^2 tissue culture flasks in supplemented MEM in a 37°C, 5% CO_2 incubator. The monolayers in each flask were infected with VSV (2.5×10^5 plaque forming unit (PFU)) in 3 mL supplemented minimal

essential medium (MEM) for MDBK: 5 x 10^5 PFU in 5 mL supplemented MEM for WISH). The infected flasks were incubated for 60 min at 37°C and were shaken every 15 min. The medium was poured out and 20 mL supplemented MEM was added to each infected flask and then incubated overnight at 37°C. When the cells were around 20% confluent, the infected flasks were frozen at -70°C. To release the VSV from the cells, the medium was thawed at room temperature. The supernatants were transferred to a 50 mL centrifuge tube and centrifuged at 500 rpm for 10 minutes. The virus-rich supernatants were pooled and placed on ice. The virus stock was aliquot into 500 L/vial and stored at -70°C. These VSV stocks were used for the antiviral assay. A single cell suspension of MDBK cells was prepared from a confluent culture and the MDBK cells were resuspended at 6-7 x 10^5 cells/mL in supplemented MEM. 50μL of supplemented MEM was added to each well of a 96-well flat-bottomed microtiter plate. 50μL of IFN standard was added to well 3 of row A and B. 50μL of the first sample was added to well 3 of row C and D, and repeated for samples 2 and 3. A 2-fold serial dilution of IFN samples was prepared by gently mixing the contents of well 3 and then transferring 50μL from well 3 to well 4, then from 4 to well 5, etc., through well 12. 50μL of sample was discarded from well 12. At this point, each well contained 50μL. 50μL cells were added to each well and the plate was agitated to ensure the cells were evenly distributed. The plate was incubated overnight at 37°C. The VSV was diluted with supplemented MEM to 10000 PFU/mL. The supernatants were aspirated from each well of the microtiter plate with an 8-channel pipettor. Each well in vertical row 1 had 100 mL supplemented MEM added. 100 mL virus was added to each well, starting with row 2 (1000 PFU/well). The plate was incubated 20-24 hours at 37°C. The monolayers were examined, prior to washing and fixing the cells, using an inverted microscope. In the cell control wells, uniform monolayers were observed. In the virus control wells, the monolayers were completely destroyed. The supernatants were removed from each well. Each well was washed three times with 100 mL cold HBSS. The final wash was aspirated and replaced with 100 mL of 5% formalin in each well and incubated 10 minutes at room temperature. The formalin was shaken from the plate into a sink with the water running. 100μL of 0.05% Crystal Violet in 20% ethanol was added to each well and incubated for 10 minutes at room temperature. The plate was rinsed with tap water, inverted on absorbent paper and allowed to dry. The samples were read spectrophotometrically, by adding 100μL of 100% methanol to each well of the plate.

The plate was agitated to elute the dye from the fixed cells. The absorbance was read at 595 nm.

Standards used in the bioassay: IFN-α 100 U/mL or 800 U/mL. Interferon-α standard diluted in serum (800 U/mL or 100U/mL).

D. ELISA Assay

To determine IFN-α concentration in skin homogenates, Cytoscreen™ ELISA kit (Medicorp, Montreal, PQ) was used. The sensitivity of the assay is <25 pg/mL and the range of concentration is 0-500 pg/mL. The assay is specific for human IFN-α with no cross-reactivity with human IFNβ, IFNγ or IFNω. The results are expressed as pg IFN-α/mg skin.

E. 2-5A Synthetase Assay

2-5A synthetase was determined by a ^{125}I-2-5A radioimmuno assay kit (Eiken Chemical Corp., Tokyo, Japan) using rabbit anti-human 2-5A antibody and goat antirabbit IgG as secondary antibody. Briefly, 2-5A synthetase was extracted from the samples by poly (I) poly (C) agarose for 10 min. After the addition of ATP solution (24μg/mL) and incubation, 100μL ^{125}I-2-5A was added, followed by the primary and secondary antibody. Radioactivity bound was determined by gamma counting. 2-5A synthetase is measured as pmol 2-5A produced/100mL/h and subsequently expressed as nmole enzyme/mg protein 1 h in skin homogenate. All samples were run in duplicates.

F. Immunohistochemistry

To demonstrate IFNα-specific immunostaining, paraformaldehyde fixed tissues were processed for parafilm embedding. Sections 5 μm in thickness were cut and after antigen retrieval with 10mM Na-citrate the sections were incubated with mouse anti-human interferon alpha antibody (1:2,000-1:5,000), which were in turn treated with a biotinylated rabbit anti-mouse secondary antibody and stained by the Avidin Biotin Complex (ABC) method.

To demonstrate the anti-proliferative effect of IFNα, paraformaldehyde fixed skin sections were selectively stained for Ki67 nuclear protein in proliferating cells using anti-M1B1 monoclonal antibodies, according to Rose, *et al. J. Clin. Pathol.*, 47:1010 (1994).

EXAMPLE 21

In vivo Administration of IFN-α to Guinea Pigs

Guinea pigs (n=3/group) were shaved with an electric razor 24 hours prior to the application of a skin patch containing a test formulation. The skin was lightly washed with distilled water and patted dry with tissue prior to the addition of the patch. The patch was applied by removing the backing paper from the adhesive foam and firmly pressing the patch to a clean area of skin away from any skin abrasion and located in a position that the animal is unlikely to access. The patch was covered with OPSITE™ occlusive dressing and a plastic tape to keep the patch in place for 24 hours. The patching was carried out under anesthesia with halothane. After treatment the patch was removed under anesthesia. Any remaining formulation was collected for analysis and the skin was checked for general condition.

After patch removal, the skin surface was cleaned by wiping the area once with a dry tissue, 4 x with a tissue soaked in 70% ethanol, 4 swabs with 0.5% (v/v) Tween 80 in distilled water using a cotton wool swab, finally 4 x with a tissue soaked in 70% ethanol. The section of the skin marked as treatment area was removed using clean sharp scissors, ensuring that only the treated area was sampled. The skin samples were frozen until analysis.

The animals were killed by cardiac puncture. Blood was collected into vacutainer tubes and centrifuged immediately. Serum was collected and aliquots were stored at -80°C until used.

The frozen skin samples were weighed and pulverized in liquid N_2 by five blows from a hammer in a tissue pulveriser (preincubated in liquid N_2). The pulverized tissue was reweighed to calculate the recovery of material and extracted by moderate vortexing with 5-10 volumes of PBS containing 1 mg/mL leupeptin and 20 mg/mL soybean trypsin inhibitor as protease inhibitors. The skin was resuspended in buffer and then sonicated 3 × 15 seconds, with 1 min intervals on ice, then centrifuged at 500g for 10 min at 4°C to remove undisrupted cells and connective tissue. The resulting supernatants were termed "whole cell homogenates" and were used immediately or aliquoted into 2-300 mL aliquots and stored at -80°C. Skin homogenates were used to determine IFN-α absorption by antiviral assay described above in the Methods section. Results are shown in Figs. 1A-1B.

EXAMPLE 22

In vivo Administration of IFN-α to Humans

A. Biphasic Lipid Vesicle Preparation

An anhydrous lipid gel was prepared by mixing the following components together:

Component	Amount (% w/w)
Phosphatidylcholine[1]	10
Cholesterol	2
monolauroyl lysine[2]	2
Propylene glycol	7

[1]Phospholipon®90H. Rhone Poulenc Roher American Lecithin Company. Dunbury CT

[2]see Table 1A for the chemical name and structure

The lipids and the solvents were weighed into a glass container and warmed to 65-75°C by intermittent heating. and gently mixed.

An oil-in-water emulsion was prepared by combining the following hydrophilic ingredients in a container and combining the following lipophilic ingredients in another container:

Hydrophilic Ingredients	Amount (% w/w)
distilled water	q.s. to 100
PEFA[1]	4.0
Methylparaben	0.15
Propylparaben	0.05
DOWICIL[2]	0.05
Cetyl alcohol	0.6
Lipophilic Ingredients	
Canola oil	4.0
Glyceryl monostearate	1.0
Synthetic beeswax	0.28

[1]PEFA = linoleamidopropyl propylene glycol-dimonium chloride

[2]Dowicil = 1-(3-chlorallyl)-3,4,7-triaza-1-azoniaadamantane chloride

The oil-in-water emulsion was prepared by adding the lipophilic mixture to the hydrophilic ingredient mixture at 60-80°C in a homogenizer at 20-80 psig for 5-30 minutes to obtain a small droplet size of less than about 0.5 μm.

In separate containers, aqueous solutions of IFN-α were prepared having activities of 5 MU, 15 MU and 40 MU. Lyophilized IFN-α (Intron A) was dissolved in part of the formulation water to the total quantity required for the batch size.

Biphasic lipid vesicles were prepared by simultaneously adding the oil-in-water emulsion and an IFN-α aqueous solution at a selected concentration to the lipid gel, which was warmed to 55°C. The lipid gel, IFN-α solution, and emulsion were vigorously mixed by vortexing or propeller mixing to achieve the desired particle size.

B. Transdermal Patch

The biphasic lipid vesicle preparations were placed in transdermal devices constructed from a backing member peripherally joined to a styrofoam adhesive member. The patches had a 5 cm outer diameter and a 3 cm inner diameter to give an active delivery area of about 7 cm^2. One gram of formulation was loaded into each patch.

C. *In vivo* Administration to Humans

1. Phase I

Seventeen human volunteers were randomized into three treatment groups. In Phase I of the study, all volunteers in each group were treated with a placebo transdermal patch containing biphasic lipid vesicles of the composition described above with no IFN-α. The patches were adhesively applied to the inner upper arm and covered with OPSITE transparent adhesive for added protection. After the 48 hour test period, 6 mm punch biopsies and blood samples were collected from each subject for analysis.

Skin sites for biopsies were prepared by removing any residual cream by thorough wiping with tissue paper and swabbing with 70% alcohol followed by local anesthesia with 1% lidocaine-epinephrine solution.

Biopsy samples were used for immunohistochemistry and for homogenate preparation for antiviral, ELISA and 2-5 A synthetase assays, and procedures for each are provided above in Example 20. Blood samples were used to prepare serum for the antiviral and ELISA assays, and to extract peripheral blood mononuclear cells (PBMC) for the 2-5 A synthetase assay. Results are shown in Figs. 2A-2C, 3A-3C and Tables 5 and 6.

2. Phase II

The human volunteers randomized during Phase I of the study were each treatment with a transdermal patch containing a biphasic delivery system of IFN-α, 5, 15 or 40 MU/g dose, for 48 hours. The patch was applied to the upper inner arm as described above. After the 48 hour test period, 6 mm punch biopsies and blood samples were collected as described above from each subject for analysis. In the test group treated with patches containing 40 MU/g skin biopsies were collected from untreated skin sites for analysis. Results are shown in Figs. 4A-4C and 5A-5C and Tables 7 and 8.

EXAMPLE 23

Biphasic Lipid Vesicle Preparation Containing Insulin

Biphasic lipid vesicles having entrapped insulin were prepared as follows. An anhydrous lipid gel was prepared by mixing the following components together:

Component	Amount (% w/w)
Phosphatidylcholine[1]	14
Cholesterol	4
Acylated amino acid[2]	1
Propylene glycol	13

[1]Phospholipon®90H, Rhone Poulenc Rorer American Lecithin Company, Danbury CT

[2]The acylated amino acids PDM_1, PDM_2, PDM_{17} and PDM_{27} were used. See Tables 1A-1F for structure and chemical names.

The lipids and the solvents were weighed into a glass container and warmed to 65-75°C by intermittent heating, and gently mixed.

An oil-in-water emulsion was prepared by combining the hydrophilic ingredients in a container and combining the lipophilic ingredients in another container:

Hydrophilic Ingredients	Amount (% w/w)
Distilled water	q.s. to 100
Phospholipid EFA™[1]	0.5
Methylparaben	0.04
Propylparaben	0.01
Lipophilic Ingredients	
olive oil	0.25

[1]linolcamidopropyl propylene glycol-dimonium chloride, DEBRO (Mississauga, Ontario)

The oil-in-water emulsion was prepared by adding the lipophilic mixture to the hydrophilic ingredient mixture at 60-80°C in a homogenizer at 20-80 psig for 5-30 minutes to obtain a small droplet size of less than about 0.5 μm.

An aqueous solution of insulin in 3 mM HCl, pH 3-3.5 was prepared at concentrations sufficient to obtain 10 mg insulin/200 mg formulation and 50 mg insulin/200 mg formulation.

Biphasic lipid vesicles were prepared by simultaneously adding the oil-in-water emulsion and the aqueous solution containing insulin to the lipid gel, which was warmed to 55°C. The gel, insulin, and emulsion were vigorously mixed by vortexing or propeller mixing to achieve the desired particle size. The pH of the formulation was adjusted with 1 M NaOH to between 4.5-5.5.

A. Transdermal Patch Preparation

The biphasic lipid vesicle preparations were placed in transdermal devices constructed from a backing member peripherally joined to a styrofoam adhesive member. The square patches were 2.25 cm^2 (1.5 cm x 1.5 cm) in size with an active delivery area of 1 cm^2. 200 mg of each biphasic lipid vesicle preparation was placed in the reservoir of each patch, except for the vesicles having 10 mg insulin/g formulation ("PDM_{27}/10mg" (see Table 3)) where 1000 mg of formulation was placed in each patch.

EXAMPLE 24

Transdermal Administration of Insulin *In vivo*

Materials

Sprague-Dawley rats (150-225g) were obtained from Charles River Laboratories

(Quebec. Canada). Streptozotocin (STZ: mixed anomers: 75% -anomer) was obtained from Sigma. Insulin was purchased from Cansera (recombinant human insulin) or from Sigma (porcine insulin). One Touch Profile blood glucose monitoring system and One Touch glucose test strips were obtained from Lifescan. Opsite was obtained from Smith & Nephew (U.K.) and Insulin ELISA kits were obtained from Mercodia (ALPCO, USA).

Insulin was originally dissolved in ~3 mM HCl, pH 3-3.5, before being adjusted to the formulation pH by 1 M - concentrated NaOH.

1. Diabetic rat model

An insulin dependent diabetes mellitus (IDDM) animal model was established to study the delivery of insulin after topical application. Sprague-Dawley rats (150-225g) were fasted for 24 hours to increase the sensitivity of the pancreatic -cells to destruction by streptozotocin (STZ). After the 24 hour fast, the rats were injected with STZ (50-55 mg/kg body weight) in 100 mM sodium citrate buffer, pH 4.5. Control rats were injected with citrate buffer alone. The rats were allowed three days for the diabetic state to stabilize. Blood glucose, body weight, and general health were recorded daily for each rat. Rats receiving topically applied insulin treatment were carefully shaved under halothane anesthesia and the skin allowed to recover for 6-24 hours.

2. Application of formulations and dosage

A. Animal Treatment. After the three-day stabilization period, the rats were divided into groups depending on the treatment to be received: (1) control, no STZ, no treatment; (2) control, STZ-IDDM, no treatment; (3) STZ-IDDM, + subcutaneous injection (1 mg insulin solution: 28 U); and (4) STZ-IDDM, + a selected transdermal formulation (10-50 mg insulin/g formulation: 280 U insulin/rat).

Those rats receiving transdermal insulin treatment were carefully shaved under anesthetic. The skin was allowed to recover for 6-24 hours prior to insulin treatment. Rats were kept with free access to food (77% carbohydrate, 14% fat, 9% protein) and water.

Insulin treatment was for three days, at the end of which the rats were sacrificed and blood and skin samples were collected.

3. Analysis of insulin levels in skin and plasma

Skin samples from the transdermal insulin-treated rats were collected as follows. The skin was washed with distilled water, swabbed 6 × with 0.5% (v/v) Tween 80 and

rinsed with distilled water and dried, followed by stripping the uppermost layer of stratum corneum by a single application of SCOTCH™ tape. This process removed any lipid vesicles or non-absorbed insulin from the surface of the skin. Half the skin samples were homogenised by freezing in liquid nitrogen and disrupting with a Bio-pulverisor (6-8 blows). The skin powder was resuspended in 5 volumes of PBS, mixed well and centrifuged to remove cellular debris. The homogenates were used immediately or stored at -20°C. The remaining half of the skin samples were incubated in 4% paraformaldehyde overnight and embedded in paraffin for immunohistochemistry.

Plasma and serum were produced by centrifuging heparinized blood. The serum was used immediately or aliquoted and stored at -20°C until used.

A. *ELISA*

Skin homogenates and serum samples (5-100 μL) were analyzed for human and porcine insulin content by ELISA (ALPCO, USA). The ELISA kit for human insulin detection had a 500% cross-reactivity with porcine insulin, however, the rat insulin cross-reactivity was 0.5% making this an accurate way to determine the specific absorption of porcine insulin in the rat model. The sensitivity of the kit was >25 pg/mL porcine insulin with a standard curve range of 50-1000 pg/mL. Human insulin detection used a superconjugate with a standard curve range of 0.15-100μIU/mL (5.36-357 pg insulin/mL) and 5-25μL of sample.

The control (no insulin) animals had a "normal" baseline insulin concentration above the reported physiological levels: control (normal) levels: 10.6 $\pm$ 6.7 pmol/mL (n = 5). The diabetic animals left untreated had insulin concetration levels of 7.6 $\pm$ 3.3 pmol/mL (n = 9).

B. *Confocal Laser Scanning Microscopy.* After being frozen in liquid nitrogen the skin was carefully cross sectioned into thin slices of approximately 0.5 mm thickness using a single edged blade. This mechanical cross-section has the advantage to visualize the fluorescence in the stratum corneum, viable epidermis and dermis in a single image. The skin was positioned with the stratum corneum parallel to a #0 cover slip (Carolina Biologicals) in an special glass holder and scanned 10 μm below the cutting surface (to avoid interference by fluorescence from damaged cell) using a Multiprobe 2001 CLSM (Molecular Dynamics, CA) equipped with an argon/krypton mixed gas ion laser and

mounted on a Nikon Diaphot cross-epifluorescence inverted microscope. A 488 nm argon/krypton laser line (20 mW or 37mW) directed through a 510 primary dichromatic filter attenuated with 1% or 3% neutral density filter was used for green fluorochromes. The pinhole size was set at 200 μm. The image size was 1024 x 1024 pixels with a pixel size of 0.6 μm for the 20x objective. A Silicon Graphics Indy computer with the software provided (Image Space) was used for both data acquisition and analysis.

4. Evaluation of the pharmacodynamic response

Blood glucose levels were recorded periodically (approx. every 4 hours) after administration of the topical dose of insulin. Blood glucose was measured by a LIFESCAN™ blood glucose meter using approximately 50μL of fresh whole blood obtained from the tail veins of the rats. Where blood insulin levels were to be determined, 0.1-0.5 mL blood was collected in labeled microfuge tubes containing 1 μg heparin. Normal blood glucose is below 8 mmol/L (above this level glucose is excreted from the kidneys); during a 24 hour period normal blood glucose fluctuates between 3.0-7.5 mmol/L. The blood glucose in diabetic subjects typically fluctuates in the range of 18-24 mmol/L. Returning, and possibly holding, the blood glucose to normal levels (3.5-7.5 mM) is indicative of successful (therapeutic) insulin treatment of diabetes insulin.

Although the invention has been described with respect to particular embodiments, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A composition, comprising:

a therapeutic agent; and

an acylated amino acid;

wherein application of said composition to the skin is effective to achieve enhanced uptake of the therapeutic agent when compared to transdermal uptake of the therapeutic agent in the absence of the acylated amino acid.

2. The composition of claim 1, further comprising a suspension of liposomes.

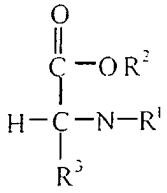
3. The composition of claim 2, wherein said therapeutic agent is entrapped in said liposomes.

4. The composition of claim 2, wherein the acylated amino acid is entrapped in said liposomes.

5. The composition of claim 2, wherein said therapeutic agent and said acylated amino acid are entrapped in said liposomes.

6. The composition of claim 5, wherein said liposomes include an oil-in-water emulsion in liposomes' central compartment.

7. The composition of claim 1, wherein the acylated amino acid represented by the formula:



wherein R^1 is an acyl group having from 1-20 carbons. R^2 is hydrogen or an alkyl group, and R^3 corresponds to a modified or unmodified R group of a selected amino acid.

8. The composition of claim 6, wherein the acylated amino acid is an acylated lysine.

9. The composition of claim 1, wherein said therapeutic agent is a protein or a nucleic acid.

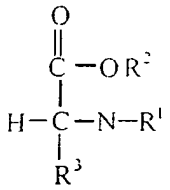
10. The composition of claim 9, wherein said protein is selected from the group consisting of an interferon, an interleukin, and insulin.

11. An interferon-α composition, comprising

biphasic lipid vesicles comprised of (i) a lipid bilayer comprising a phospholipid and a fatty acylated amino acid; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, said oil-in-water emulsion being stabilized by a surfactant; and (iii) interferon-α entrapped in said vesicles;

wherein said composition when applied to the skin of a subject being effective to administer a therapeutically effective amount of interferon-α.

12. The composition of claim 11, wherein the acylated amino acid represented by the formula:



wherein R^1 is an acyl group having from 1-20 carbons, R^2 is hydrogen or an alkyl group, and R^3 corresponds to a modified or unmodified R group of a selected amino acid.

13. The composition of claim 12, wherein R^2 is $((CO)C_{19}H_{39})$.

14. The composition of claim 13, wherein said amino acid is serine or threonine.

15. The composition of claim 12, wherein said fatty acylated amino acid is monolauroyl lysine.

16. Use of a composition according to any one of claims 11-15 for treating human papilloma virus in a subject.

17. A composition for transdermal administration of insulin, comprising biphasic lipid vesicles comprising (i) a lipid bilayer comprised of a phospholipid and a fatty acylated amino acid; (ii) an oil-in-water emulsion entrapped in the biphasic lipid vesicles, said oil-in-water emulsion comprised of a triglyceride dispersed in a water phase and stabilized by a surfactant; and (iii) insulin entrapped in said vesicles; said composition when applied to the skin of a subject being effective to administer a therapeutically effective amount of insulin.

18. The composition of claim 17, wherein said lipid bilayer is further comprised of a sterol.

19. The composition of claim 17, wherein said acylated amino acid is an acylated lysine.

20. The composition of claim 19, wherein said acylated lysine is Nα-capryloyl-Nε lauroyl L-lysine ethyl ester.

21. Use of a composition according to any one of claims 17-20 for treatment of diabetes in a human subject.

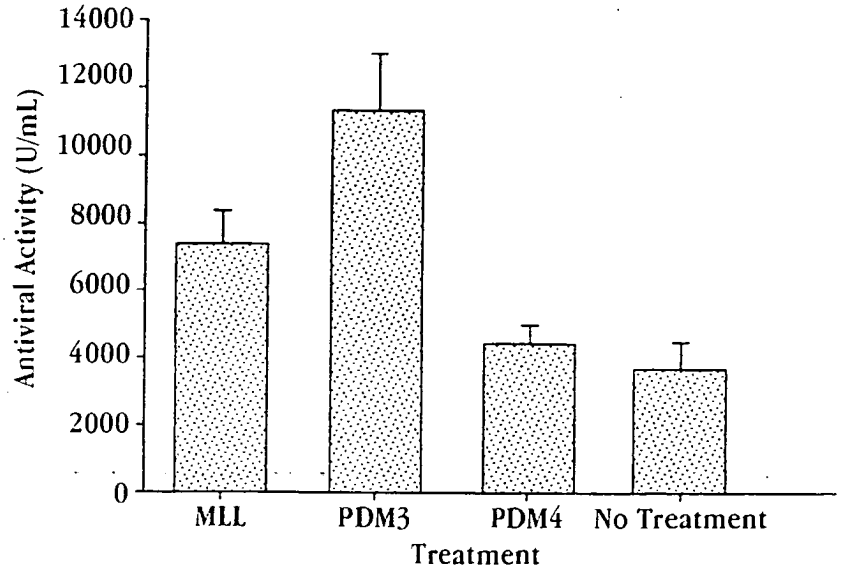

14000
12000
10000
8000
6000
4000
2000
0
Antiviral Activity (U/mL)
MLL
PDM3
PDM4
No Treatment
Treatment

Fig. 1A

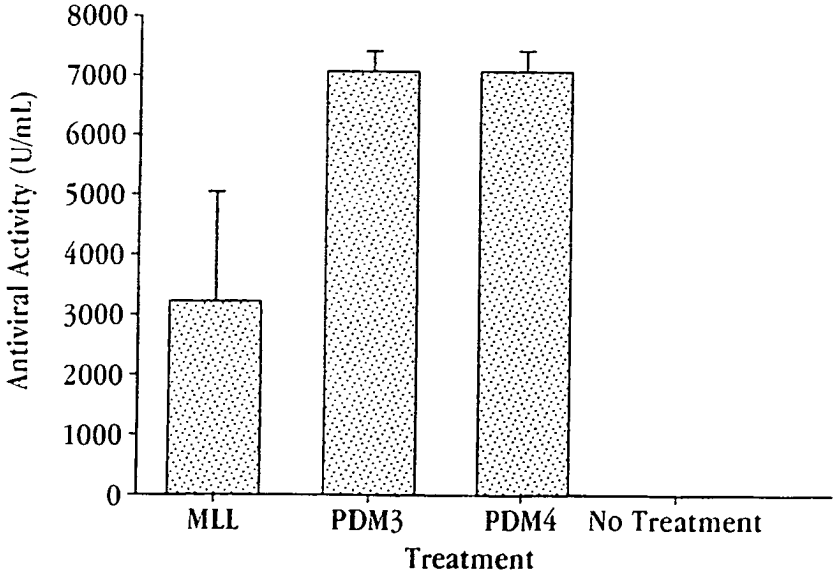

8000
7000
6000
5000
4000
3000
2000
1000
0
Antiviral Activity (U/mL)
MLL
PDM3
PDM4
No Treatment
Treatment

Fig. 1B

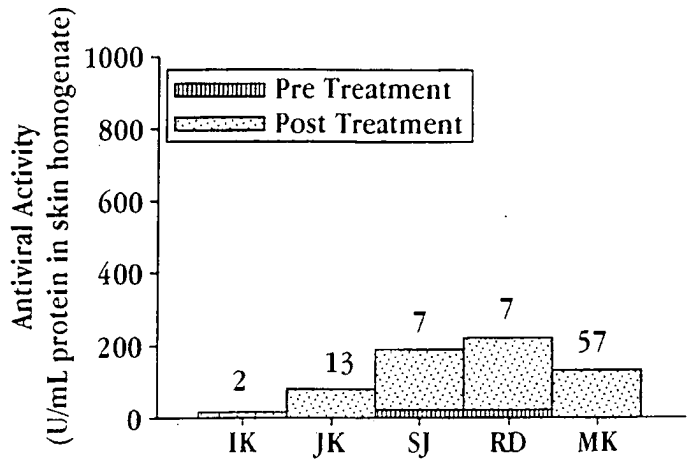


Fig. 2A

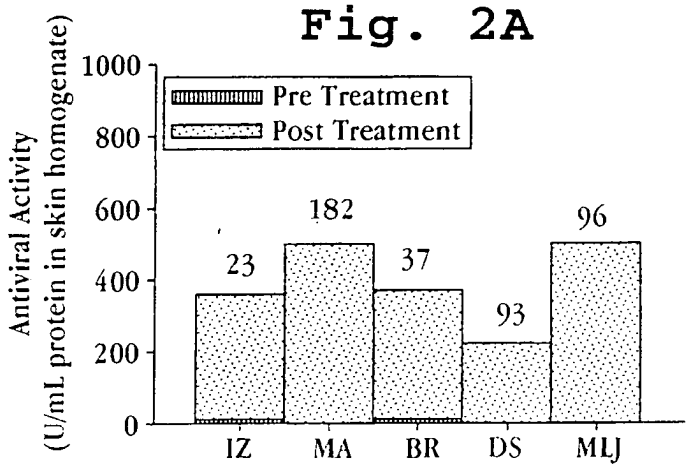


Fig. 2B

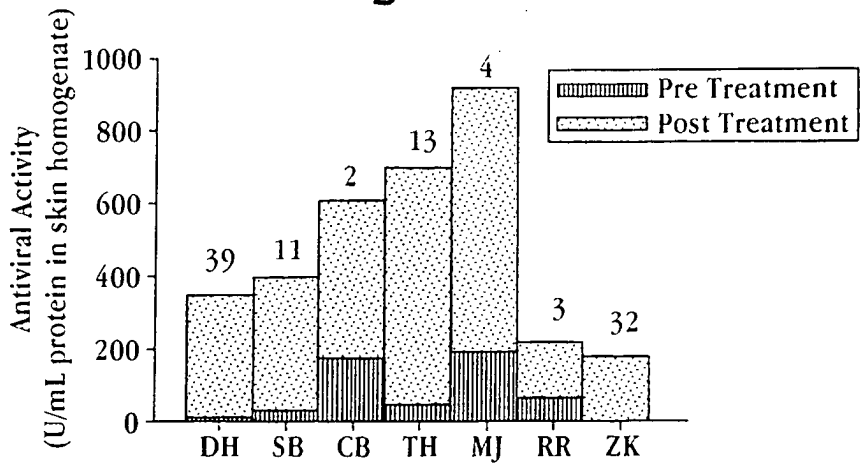


Fig. 2C

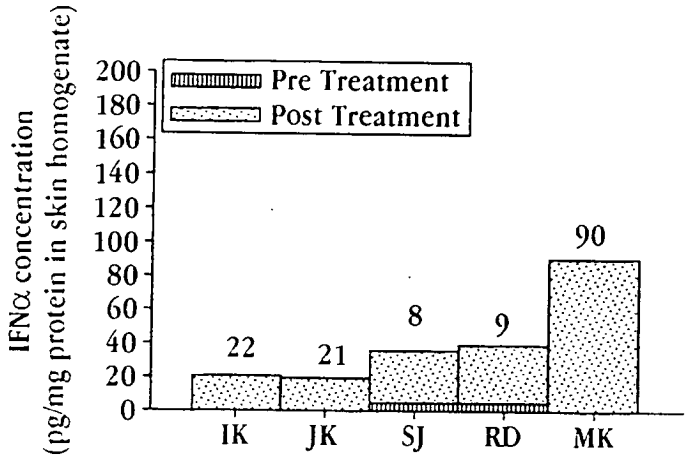


Fig. 3A

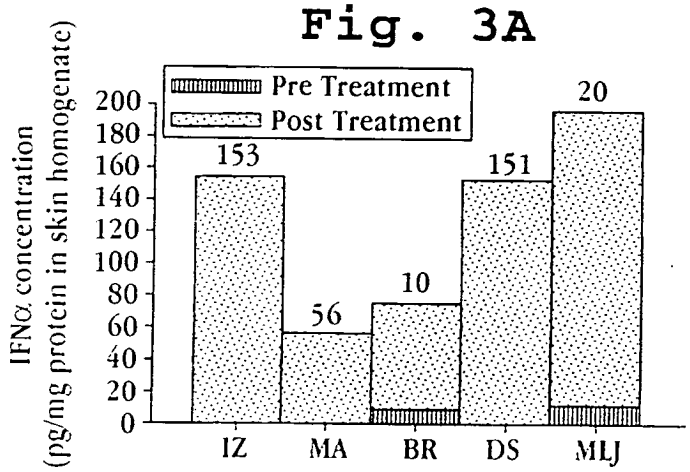


Fig. 3B

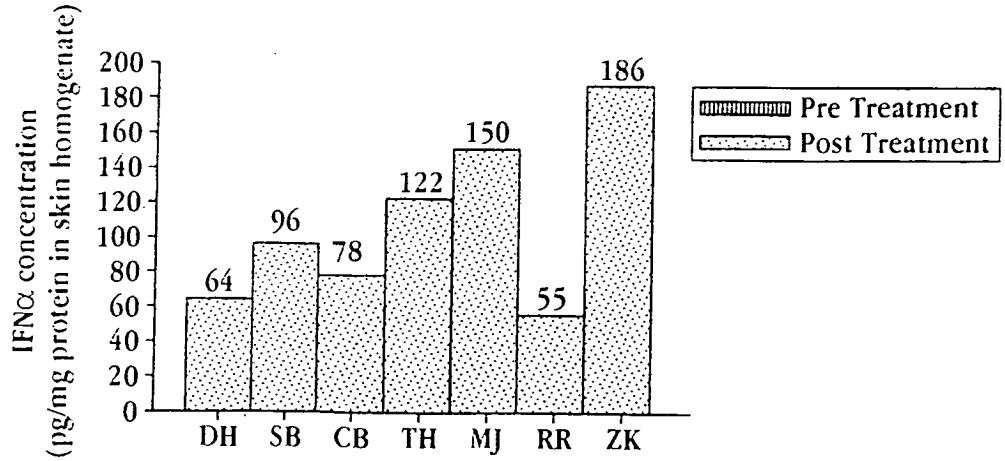


Fig. 3C

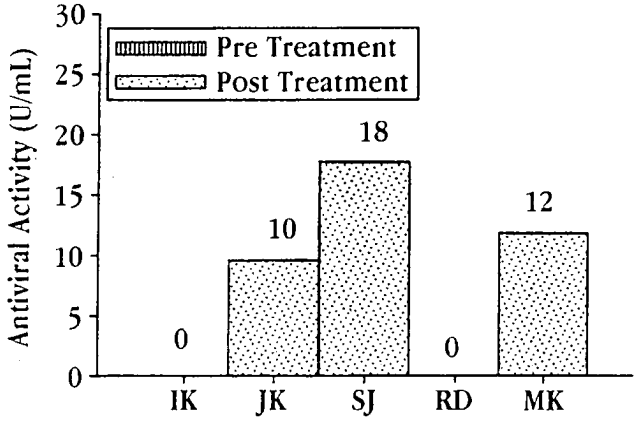


Fig. 4A

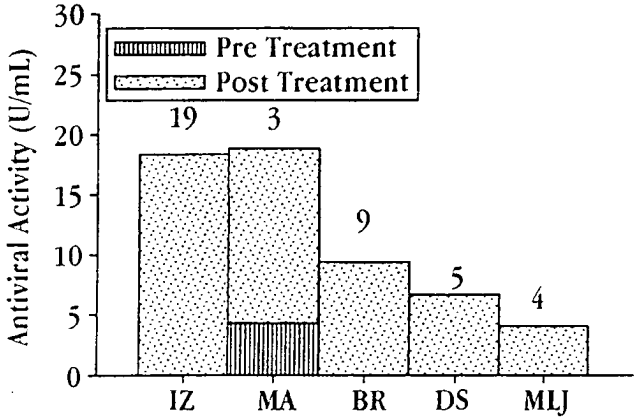


Fig. 4B

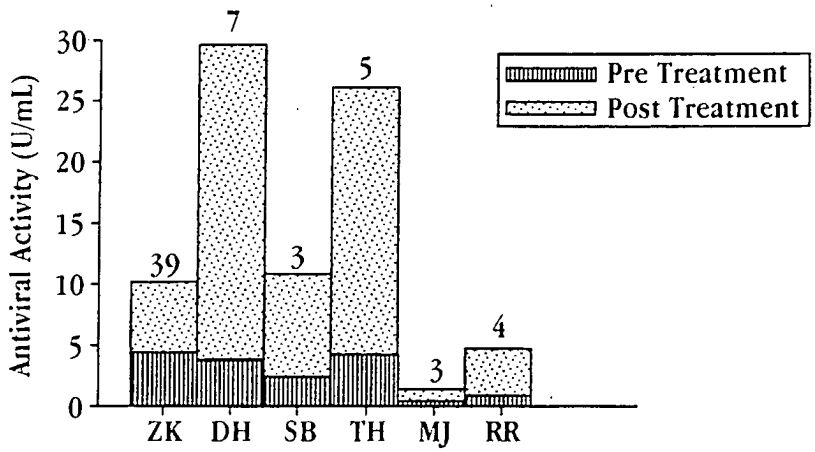


Fig. 4C

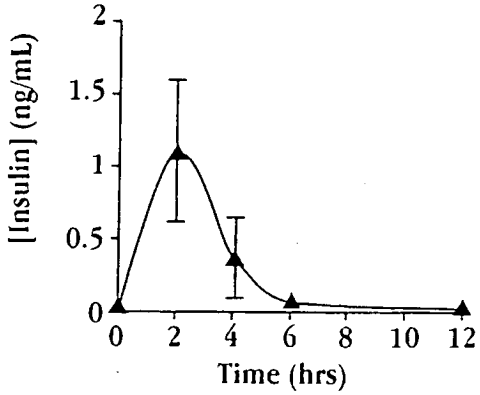


Fig. 6

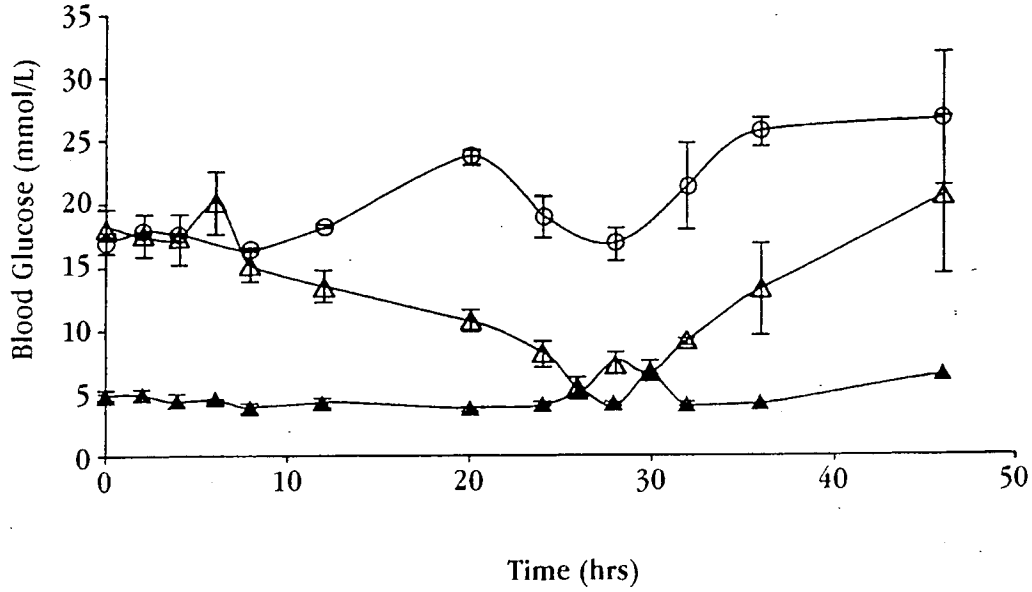


Fig. 7

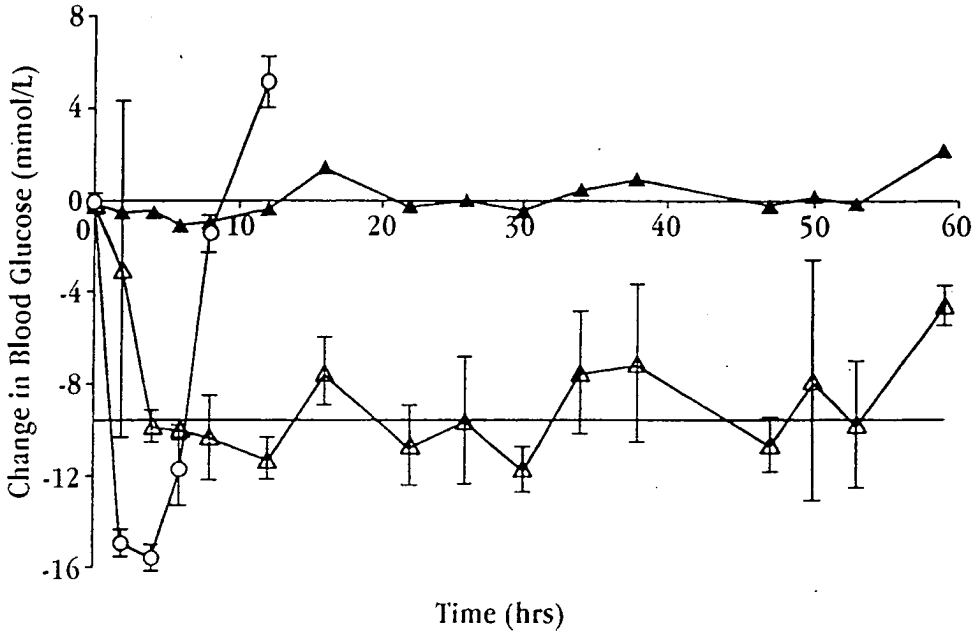

8
4
0
-4
-8
-12
-16
0
10
20
30
40
50
60
Change in Blood Glucose (mmol/L)
Time (hrs)

Fig. 8

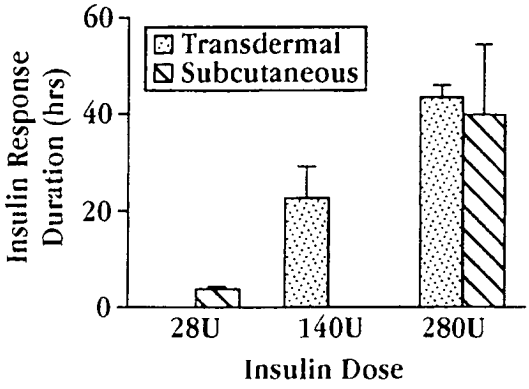

60
40
20
0
Transdermal
Subcutaneous
Insulin Response
Duration (hrs)
28U
140U
280U
Insulin Dose

Fig. 9

INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/CA 00/01323

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/18 A61K9/127 A61K9/70 A61P3/10 A61P31/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L,X	US 5 853 755 A (FOLDVARI MARIANNA) 29 December 1998 (1998-12-29) cited in the application	1-12,15, 16
Y	Document so quoted for its casting doubt on the validity of the convention-priority claim. column 3, line 24 - line 59 column 7, line 61 - line 47 column 13, line 16 - line 19; examples 4,6,8,10-13,16.17 column 29 -column 30; claims 1,6,7,9-12,14; table 10	17-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2001	08/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Marttin, E

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Int. ational Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 552 405 A (LINTEC CORP) 28 July 1993 (1993-07-28) page 3, line 1 -page 4, line 36 page 4, line 48 -page 5, line 24 page 6, line 30 - line 47; claims 1-5; examples 1-7; tables 1,2	1,7
X	EP 0 364 211 A (SEKISUI CHEMICAL CO LTD) 18 April 1990 (1990-04-18) page 4, line 18 - line 35; example 4; table 1 page 13, line 15 - line 50; claims 1,3; table 4	1,7
X	US 4 980 378 A (WONG OOI ET AL) 25 December 1990 (1990-12-25) column 4, line 30 - line 63 column 9, line 33 - line 35; claim 1; examples 2,5,6; tables 1,2	1,7
Y	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; GAMA, YASUO ET AL: "Manufacture of controlled-release liposomes containing N,N-diacylcystine as the membrane material" retrieved from STN Database accession no. 117:118493 XP002160920 abstract & JP 04 074116 A (KOGYO GIJUTSUIN, JAPAN;LINTEC K. K.) 9 March 1992 (1992-03-09) abstract	17-21
Y	US 4 670 584 A (TOYOSHIMA SHIGESHI ET AL) 2 June 1987 (1987-06-02) column 1, line 19 -column 2, line 5 column 13, line 43 -column 14, line 54; tables 2,3 column 15, line 6 -column 16, line 19; claim 1	17-21
Y	EP 0 418 642 A (TEIKOKU SEIYAKU KK ;TOYOJOZO CO LTD (JP)) 27 March 1991 (1991-03-27) page 4, line 24 - line 56 page 10, line 44 - last line; claims 1,2	17-21

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Int. ational Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 475 160 A (CEVC GREGOR) 18 March 1992 (1992-03-18) page 2, line 1 - line 13 page 3, line 9 - line 29 page 3, line 53 -page 4, line 16; claims 1,15-21,23; examples 166,236-243	17-21

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